

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

In re Patent Application of	)	
	)	
Ming-Bo Wang et al.	)	Group Art Unit: 1635
	)	
Application No.: 09/287,632	)	Examiner: JANE J ZARA
	)	
Filed: April 7, 1999	)	Confirmation No.: 6526
	)	
For: METHODS AND MEANS FOR	)	
OBTAINING MODIFIED PHENOTYPES	)	
	)	
	)	

**DECLARATION BY INVENTOR UNDER 37 C.F.R. § 1.131**

Commissioner for Patents  
P.O. Box 1450  
Alexandria, VA 22313-1450

Sir:

We, Peter Michael Waterhouse, Ming-Bo Wang, Michael Wayne Graham and Neil A. Smith, citizens of Australia, hereby state as follows:

1. We are the named inventors of the subject matter of the above captioned application.

2. We have read and understood the Office Action dated November 1, 2007. We understand that U.S. Patent No. 6,506,559 has been cited in the Office Action in rejecting the claims of the present application.

3. We submit Exhibits 1-5 as evidence that embodiments of the presently claimed invention were made, i.e. actually reduced to practice, prior to the December 23, 1997 filing date of U.S. Provisional Application No. 60/068,562 of which benefit is claimed by U.S. Patent No. 6,506,559.

4. Exhibit 1 is a copy of 7 pages from a laboratory notebook prepared by Neil Smith that recorded experiments performed by Neil Smith and/or under his supervision during the construction of a chimeric DNA comprising two copies of a 0.75 kb fragment of the cDNA copy

of the Potato Virus Y RNA genome (PVY) in inverted orientation under control of a CaMV 35S promoter and further comprising intron 2 of the *Pdk* gene from *Flaveria trinervia* in the transcribed region of the chimeric DNA.

5. Notebook page 151 (Exhibit 1, page 1) describes the relevant part of expression vector N2, which was used as starting material to produce the chimeric DNA as described in paragraph 4. That N2 is an expression vector can be seen from the notation ART7, which indicates the backbone of the expression vector pART7 which was commonly used within the CSIRO Plant Industry laboratories at that time. The figure indicates the restriction enzyme sites and denotes the different functional parts of the N2 vector as blocks. The bent arrows indicate the promoter orientation, while the straight arrows indicate the orientation of the PVY inserts. The sizes of the functional parts are indicated between brackets below the blocks and are noted as kilobasepairs. Abbreviations used are: 35S: Cauliflower Mosaic Virus 35S promoter; PVY: approximately 0.75kb fragment of the sequence encoding the protease of potato virus Y; S4: 0.6 kb fragment of the Subterranean Clover Stunt Virus segment S4; Ocs3': 3' termination and polyadenylation signal from the octopine synthase gene from *Agrobacterium tumefaciens*.

6. The notebook page 151 further indicates a first cloning strategy to produce a PVY hairpin RNA having complementary sense and antisense strands targeting the PVY genome part, whereby the 0.6kb HindIII fragment comprising the Subterranean Clover Stunt Virus segment S4 fragment was deleted. In the product, both copies of the PVY fragment are operably linked under the control of one CaMV35S promoter. Upon transcription from the CaMV35S promoter which is active in plant cells, a double stranded RNA molecule as schematically represented ("mRNA double stranded") would be produced. The RNA molecule as represented is an artificial hairpin RNA structure comprising two annealing RNA sequences labeled "sense strand" and " $\alpha$  sense strand", wherein one of the annealing RNA sequences comprises a sense sequence of 750 nucleotides identical to 750 consecutive nucleotides of a PVY target gene of interest in a eukaryotic cell, in this case a plant cell, and the second of the annealing RNA sequences comprises an antisense sequence identical to 750 consecutive nucleotides of the complement of the PVY target gene of interest.

7. Notebook pages 186-188 (Exhibit 1, pages 2-4) describe the preparation of intron 2 of the *pdk* gene of *Flaveria trinervia* and cloning of a PCR amplified fragment having intron 2 (767bp) flanked by restriction enzymes into a small cloning vector (PBC) for amplification purposes.

8. On Notebook page 186 plasmid *pdk1* is described comprising a 10.5 kb *EcoRI* fragment of the *pdk* genomic DNA in pBLUESCRIPT vector. The figure is a schematic representation of the *pdkA* genomic DNA, the cloned *EcoRI* fragment and the position of intron 2. At the bottom is a photo of diagnostic restriction enzyme digests to confirm the structure of *pdk1*.

9. Notebook page 187 represents a nucleotide sequence alignment of the *pdkA* and *pdkB* genes with an indication of the position and nucleotide sequence of intron 2 of *pdkA*. Further indicated are the nucleotide sequences and position of the oligonucleotides used as PCR primers to specifically amplify the intron 2 fragment, flanked by *HindIII* restriction enzyme recognition sites.

10. Notebook page 188 details the PCR reaction protocol to amplify the intron 2 fragment from the plasmid *pdk1*, a gel analysis of the amplified fragment, a ligation protocol for introduction of the PCR- amplified, *HindIII* restricted product into a small vector named PBC and determination of the relative orientation of insertion of the *HindIII* fragment comprising the intron 2 by restriction analysis. At the bottom of the page, the two alternative orientations of insertion into PBC are schematically represented, together with an indication of the expected size of the fragment which was generated after *BglII*/*PstI* restriction digestion for both orientations and indication of the orientation of inserts in the different clones.

11. The first picture of the gel on Notebook page 189 concerns additional verification for the clones of the intron 2 *HindIII* fragment in PBC in both orientations. Immediately below the

picture is a schematic representation of the cloning strategy to introduce the intron 2 between the two fragments of PVY in inverted orientation under control of a CaMV35S promoter, to generate a double stranded RNA-encoding chimeric gene. To this end, the 0.6 kb HindIII S4 fragment from T-DNA vector pNS2 was replaced by the 0.77 kb HindIII fragment comprising the intron 2 described under paragraph 10. pNS2 comprised the gene components of expression vector N2 described on notebook page 151 (discussed in paragraph 5 above) cloned into T-DNA vector pART27 (see Exhibit 1, page 6 where it is indicated that the backbone vector is pART27, a T-DNA vector commonly used at CSIRO for introduction of chimeric DNAs into plant cells by Agrobacterium-mediated transformation). Notebook page 189 further describes the composition of the ligation mix and the restriction enzyme analysis to identify clones in which the S4 fragment had been replaced by the intron 2 fragment. A small table at the bottom of notebook page 189 indicates the predicted sizes of fragments generated by restriction enzyme digests for each of the two possible orientations of the inserted intron fragment (indicated as "sense intron" and "α-sense intron", the latter is shorthand for intron in antisense orientation).

12. Notebook page 192 (Exhibit 1, page 6) displays the restriction enzyme analysis with Apal enzyme to verify which of the candidate clones contained a single insert of the intron fragment. As indicated next to the gel, the clones with a single insert were expected to produce a restriction fragment of 770 bp, and as stated there "appear to be many [candidate clones] with single Intron 2 inserts". The potentially good chimeric DNA constructs were further verified through XbaI restriction enzyme analysis which as indicated were expected produce a characteristic 2.3 kb fragment. At least candidates "2 and 6 (from pool 7) appear to have [the] intron insert".

13. The bottom part of the Notebook page 192 then represents the restriction enzyme analysis with the restriction enzyme combinations indicated at the bottom of page 189 to determine the orientation of the intron 2 in the chimeric DNA constructs for 4 different clones. The notification under the gel pictures indicate that clone "2" had the intron in antisense orientation, while clones "6", "original single clone 1" and clone "6 from pool 7" had the intron fragment in the sense orientation. Intron 2 is a heterologous intron with respect to the PVY inserts in the chimeric DNA.

14. Notebook page 193 (Exhibit 1, page 7) represents a quality control of a large scale DNA preparation of the T-DNA vector clones 2 and 6 from Notebook page 192. At the top of page 193 it is indicated that the chimeric DNA construct with the intron fragment in antisense orientation is named pNS9, while the chimeric DNA construct with the intron fragment in sense orientation is named pNS10. The bullet point "set up 2 and 6 for tri-parental => LBA4404" refers to the introduction of T-DNA vectors from the E. coli host into Agrobacterium tumefaciens comprising a disarmed helper Ti-plasmid by a process referred to as triparental mating commonly used in the art. The resulting agrobacterial transconjugant strains were cocultivated with tobacco W38 cells in order to introduce the chimeric DNA into plant cells.

15. Exhibit 1 thus describes the successful construction of a chimeric DNA construct molecule comprising, in order:

- a) a promoter, operative in a eukaryotic (plant) cell (*CaMV35S*);
- b) a DNA region, which when transcribed, yields an RNA molecule with at least one RNA region with a nucleotide sequence comprising
  - i. a sense nucleotide sequence including at least 20 consecutive nucleotides (and also at least 50 or 100 nucleotides) having 100% sequence identity with at least 20 consecutive nucleotides (and also at least 50 or 100 nucleotides) of the nucleotide sequence of the nucleic acid of interest (*0.75 kb PVY region in sense orientation- the nucleic acid of interest is thus comprised in the genome of an infecting RNA virus in this embodiment*)); and
  - ii. an antisense nucleotide sequence including at least 20 consecutive nucleotides (and also at least 50 or 100 nucleotides) having 100% sequence identity with the complement of the at least 20 consecutive nucleotides (and also at least 50 or 100 nucleotides) of the sense nucleotide sequence (*0.75 kb PVY region in antisense orientation*);wherein the RNA is capable of forming an artificial hairpin RNA structure with a double stranded RNA stem by base-pairing between the regions with sense and antisense nucleotide sequences; and

wherein the DNA region further comprises an intron (*intron 2*) (which is a heterologous intron with respect to the sense and antisense PVY sequences); and

- c) a DNA region involved in transcription termination and polyadenylation (3' *ocs region*).

16. The experiments recorded in Exhibit 1 were completed prior to December 23, 1997.

17. The experiments recorded in Exhibit 1 were performed in CSIRO Plant Industry laboratories in Canberra, Australia.

18. Exhibit 2 is a copy of 5 pages from a laboratory notebook (Notebook No 5) prepared by Ming-Bo Wang that recorded experiments performed by Ming-Bo Wang and/or under his supervision during the construction of a chimeric DNA encoding an RNA comprising sense and antisense nucleotide sequences targeted to a  $\beta$ -glucuronidase gene (GUS) in which the sense and antisense nucleotide sequences could basepair over about 558 bases, as described in the above mentioned US patent application in Example 1, Figure 1A (6) and Sequence Listing entry SEQ ID NO 1. Further recorded in Exhibit 2 are the introduction of the chimeric gene into a T-DNA vector and introduction of the resulting T-DNA vector into *Agrobacterium tumefaciens* comprising a disarmed helper Ti-plasmid. The introduction of the chimeric DNA into plant cells, and data showing reduction of target gene expression in the plant cells are recorded in Exhibits 4 and 5.

19. Exhibit 2, page 1, entitled "To make invert-repeat GUS construct for rice transformation" recorded the starting materials and ligation mixes set up to introduce a second copy of part of the GUS coding region into a vector pWUJGdT comprising a GUS coding region with a deletion of the internal 0.3kb EcoRV fragment.

20. Two cloning strategies were followed i.e.

- a. to introduce a HincII fragment from the GUS coding region derived from vector pWJKKGUS (indicated on top of the page as DNA 3) into EcoRI restricted vector pWUJGdT (indicated on top of the page as DNA 1 –

treated to render the sticky ends of the restriction fragment blunt and further treated with alkaline phosphatase (AP) as indicated in the note at the top right of the page. This ligation mix is recorded as ligation 2.

- b. To introduce a *Sma*-*EcoRV* fragment from the GUS coding region derived from vector pWJKKGUS (indicated on top of the page as DNA 4) into *Sma* restricted vector pWUJGdT (indicated on top of the page as DNA 2 – treated with alkaline phosphatase (AP) as indicated in the note at the top right of the page). This ligation mix is recorded as ligation 4.

21. Exhibit 2, page 2 recorded the identification of candidate clones resulting from the cloning strategies, as well as identification of the orientation of the insert into the vector. At the bottom of the page, these two possible insert orientations into the chimeric DNA are schematically represented, resulting in two different vectors namely:

- a. candidate clone 2 (2) renamed pMBW234 where the chimeric gene comprised in order a promoter region from maize ubiquitin gene ("Ubi-P"); a 5' untranslated leader region from Johnsongrass mosaic virus ("JGMV"); a dysfunctional  $\beta$ -glucuronidase gene (GUSd) and the 5' end of the GUS coding region (GUS5') in the same orientation as the GUSd coding region, followed by transcription termination and polyadenylation region from *Agrobacterium* T-DNA gene *tml* (*tml'*).
- b. candidate clone 2 (4) renamed pMBW233 where the chimeric gene comprised in order a promoter region from maize ubiquitin gene ("Ubi-P"); a 5' untranslated leader region from Johnsongrass mosaic virus ("JGMV"); a dysfunctional  $\beta$ -glucuronidase gene (GUSd) and the 5' end of the GUS coding region (GUS5') in the inverse orientation relative to the GUSd coding region, followed by transcription termination and polyadenylation region from *Agrobacterium* T-DNA gene *tml* (*tml'*). Transcription of this chimeric gene yields an RNA molecule comprising an RNA region capable of forming an artificial hairpin RNA structure comprising two annealing RNA sequences, namely a sense RNA sequence identical to about 558 consecutive nucleotides of the GUS

gene and an antisense RNA sequence identical to about 558 consecutive nucleotides of the complement of the GUS gene.

22. Exhibit 2, page 3 records the experiments performed to clone the chimeric genes described under paragraph 21, as NotI restriction fragments, into a T-DNA vector designated binary vector pWBVec4A. As indicated in the top right insert, "ligation 2" uses "DNA 1", i.e NotI fragment from pMBW234 ; "ligation 3" uses "DNA 2" i.e NotI fragment from pMBW233.

23. Exhibit 2, page 4 contains the continued recorded data from the experiments described under paragraph 22, including the restriction enzyme analysis, of candidate clones from ligations 2 and 3 mentioned on the previous page. The plasmid denoted pMBW237 refers to the chimeric gene in colony 2(8) [clone 8 of ligation mix 2, thus corresponding to a chimeric gene as in pMBW234], and the plasmid denoted pMBW239 refers to the chimeric gene in colony 3(8) [clone 8 of ligation mix 3, thus corresponding to a chimeric gene as in pMBW233].

24. At the bottom of Exhibit 2, page 4 it is indicated that T-DNA vectors 2(8) and 3(8) were used for triparental mating, referring to the introduction of the T-DNA vectors from the *E. coli* host into *Agrobacterium tumefaciens* comprising a disarmed helper Ti-plasmid, and Exhibit 2, page 5 records the verification of the *Agrobacterium* transconjugants as containing the respective T-DNA vectors.

25. The experiments recorded in Exhibit 2 were completed prior to December 23, 1997.

26. The experiments recorded in Exhibit 2 were performed in CSIRO Plant Industry laboratories in Canberra, Australia.

27. Exhibit 3 is a copy of a publication by Alan H. Christensen and Peter H. Quail, entitled "Ubiquitin promoter-based vectors for high-level expression of selectable and/or screenable marker genes in monocotyledonous plants", which was published in *Transgenic Research* Volume 5, 213-218 (May 1996). This publication describes the ubiquitin promoter region fragment used in the construction of the chimeric genes described in Exhibit 2, as explained under paragraph 21. As indicated in Figure 1, the Ubi promoter region included an intron (Ubi-1 intron) indicated in the Figure by an angled line) in the transcribed region. This publication is mentioned in the above mentioned patent application as a reference for the Ubiquitin promoter used in the chimeric constructs described in Example 1 of that patent



application. At the time of the construction of the chimeric genes, described under paragraphs 20 and 21, we were aware of the presence of an intron in the transcribed DNA region under the control of the Ubi promoter region.

28. Exhibit 4 is a copy of 6 pages from a laboratory notebook (Notebook 96) prepared by Ming-Bo Wang that recorded experiments performed by Ming-Bo Wang and/or under his supervision to transform transgenic rice calli containing and expressing a recombinant GUS gene as a target gene of interest in a eukaryotic (plant) cell, with the T-DNA vectors described in Exhibit 2, and to analyse phenotypic expression of GUS in the supertransformed rice calli comprising the target gene and the chimeric DNAs. Data from these analyses correspond to the data recorded in the patent application as "Example 1".

29. Exhibit 4, page 1 indicates that calli were initiated from transgenic rice seeds 4R-V10-28 and 4R-V10-67, both expressing a recombinant GUS gene of interest integrated in the genome of the calli cells. This target gene is a transgene in the cells. The bottom half indicates that these calli were "supertransformed" using different Agrobacteria comprising the different T-DNA vectors including pMBW239 (experiment indicated as "7"). Note also the control experiments where an "empty" control T-DNA vector (pMBW223) was used (experiment 1) and where a conventional co-suppression construct (comprising a transcribed sense nucleotide sequence of the GUSd gene (pMBW225) was used for comparison of the level of phenotypic expression of the target gene (experiment 2).

30. Exhibit 4, page 2 recorded the data for Bialaphos resistant calli obtained with the different Agrobacteria described on the previous page (paragraph 24 above), indicating that such calli had been transformed by the T-DNA's including the chimeric DNA silencing constructs (or control constructs). The transgenic calli obtained by transformation of the initial V10-28 transgenic rice cells with pMBW239 are indicated as "7a" whereas the transgenic calli obtained by transformation of the initial V10-67 transgenic rice cells with pMBW239 are indicated as "7b".

31. Exhibit 4, page 2 at the bottom and continuing on page 3, recorded the data of an initial GUS staining experiment on parts of the transgenic calli that were obtained (V10-67 derived). In control experiment "1b" where the GUS transgenic calli were supertransformed by an "empty" T-DNA vector, all supertransformed calli strongly stained blue indicating strong GUS activity. On page 3 of Exhibit 4, the results of a similar GUS staining on calli obtained by supertransformation with pMBW239 indicating that the observed GUS staining was either weak

("W") , negative ("-") or resulted in an isolated blue spot. These data indicated that the chimeric DNA from pMBW239 reduced the phenotypic expression of the GUS target gene in the rice cells.

32. Exhibit 4, pages 4 and 5 represent the recordation of these data for the individual calli. Exhibit 4, page 6 summarizes the data of an initial GUS staining experiment on parts of the obtained transgenic calli (V10-28 derived). While in series 1a, all calli transformed by the control T-DNA vector pMBW223 strongly stained blue indicating strong GUS activity, the transgenic calli obtained by transformation with pMBW239 (series 7a) all scored as "very weak" or "basically GUS negative" with the exception of only two calli. On the bottom of page 6, reference is made to (quantitative) GUS assays, the data of which are recorded in Notebook 5, starting on page 55, discussed hereinafter as Exhibit 5.

33. The experiments recorded in Exhibit 4 were completed prior to December 23, 1997.

34. The experiments recorded in Exhibit 4 were performed in CSIRO Plant Industry laboratories in Canberra, Australia.

35. Exhibit 5 is a copy of 17 pages from a laboratory notebook (Notebook 5) prepared by Ming Bo Wang that recorded quantitative GUS analysis experiments performed by Ming-Bo Wang and/or under his supervision, on the supertransformed rice calli described in Exhibit 4. Particular attention is drawn to the "raw data" concerning GUS analysis by kinetic value measurements on Exhibit 5, page 14 which are represented in Example 1, Table 2 of the above mentioned patent application. See e.g. data for Plate No 3, wells 1A to 2H which have in handwriting indications "7a1" to "7a14" referring to transgenic lines, which are identical to the entries in Table 2 of the patent application, column indicated by "Inverted repeat CoP(6)", row V10-28. Similarly, data for Plate No 3, wells 3A to 4D which have in handwriting indications "7b2" to "7b16" referring to transgenic lines are identical to the entries in Table 2 of the patent application, column indicated by "Inverted repeat CoP(6)", row V10-67.

36. The experiments recorded in Exhibit 5 were completed prior to December 23, 1997.

37. The experiments recorded in Exhibit 5 were performed in CSIRO Plant Industry laboratories in Canberra, Australia.

38. The experiments recorded in Exhibit 2, 4 and 5 therefore describe successful completion prior to December 23, 1997 of the construction of a chimeric DNA molecule (**pMBW233/239 series**) comprising in order

- a. a promoter operative in a eukaryotic (plant) cell (**Ubi-P**);
- b. a DNA region, which when transcribed, yields an RNA molecule with at least one RNA region with a nucleotide sequence comprising
  - i. a sense nucleotide sequence including at least 20 consecutive nucleotides (and also at least 50 or 100 nucleotides) having 100 % sequence identity with at least 20 (and also at least 50 or 100 nucleotides) consecutive nucleotides of the nucleotide sequence of a nucleic acid of interest (**Gusd in sense orientation**) in a eukaryotic cell; and
  - ii. an antisense nucleotide sequence including at least 20 consecutive nucleotides (and also at least 50 or 100 nucleotides) having 100% sequence identity with the complement of the at least 20 consecutive nucleotides (and also at least 50 or 100 nucleotides) of the sense nucleotide sequence (**Gus5' in antisense orientation**);

wherein the RNA is capable of forming an artificial hairpin RNA structure with a double stranded RNA stem by base-pairing between the regions with sense and antisense nucleotide sequence wherein the DNA region further comprises an intron (**Ubi intron**) (which is heterologous to the sense GUS sequence); and

- c. a DNA region involved in transcription termination and polyadenylation (**tml'**).

39. The experiments recorded in Exhibit 2, 4 and 5 also describe successful completion prior to December 23, 1997 of a method for reducing the phenotypic expression of a nucleic acid of interest which is normally capable of being expressed (**GUS gene**) in a eukaryotic cell (a plant cell, **rice**) comprising the step of introducing into the eukaryotic cell (plant cell) a chimeric DNA (**pMBW233/239 series**) as described in paragraph 24.

40. The statements made in this declaration are made on the personal knowledge or on the information and belief of the declarants. We hereby declare that all statements made herein of personal knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date: 1st May 2008

Peter Michael Waterhouse  
Peter Michael Waterhouse,

Date: 1st May 2008

Ming-Bow Wang  
Ming-Bow Wang

Date: \_\_\_\_\_

\_\_\_\_\_  
Michael Wayne Graham

Date: 1st MAY 2008

Neil A. Smith  
Neil A. Smith

Attorney's Docket No. 1021565-000060

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

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Commissioner for Patents  
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Sir:

We, Peter Michael Waterhouse, Ming-Bo Wang, Michael Wayne Graham and Neil A. Smith, citizens of Australia, hereby state as follows:

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2. We have read and understood the Office Action dated November 1, 2007. We understand that U.S. Patent No. 6,506,559 has been cited in the Office Action in rejecting the claims of the present application.

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**Buchanan Ingersoll & Rooney PC**  
Attorneys & Government Relations Professionals

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Page 2

of the Potato Virus Y RNA genome (PVY) in inverted orientation under control of a CaMV 35S promoter and further comprising intron 2 of the *Pdk* gene from *Flaveria trinervia* in the transcribed region of the chimeric DNA.

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Page 3

7. Notebook pages 186-188 (Exhibit 1, pages 2-4) describe the preparation of intron 2 of the *pdK* gene of *Flaveria trinervia* and cloning of a PCR amplified fragment having intron 2 (767bp) flanked by restriction enzymes into a small cloning vector (PBC) for amplification purposes.

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9. Notebook page 187 represents a nucleotide sequence alignment of the *pdKA* and *pdKB* genes with an indication of the position and nucleotide sequence of intron 2 of *pdKA*. Further indicated are the nucleotide sequences and position of the oligonucleotides used as PCR primers to specifically amplify the intron 2 fragment, flanked by *HindIII* restriction enzyme recognition sites.

10. Notebook page 188 details the PCR reaction protocol to amplify the intron 2 fragment from the plasmid *pdK1*, a gel analysis of the amplified fragment, a ligation protocol for introduction of the PCR- amplified, *HindIII* restricted product into a small vector named PBC and determination of the relative orientation of insertion of the *HindIII* fragment comprising the intron 2 by restriction analysis. At the bottom of the page, the two alternative orientations of insertion into PBC are schematically represented, together with an indication of the expected size of the fragment which was generated after *BglII*/*PstI* restriction digestion for both orientations and indication of the orientation of inserts in the different clones.

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Page 4

picture is a schematic representation of the cloning strategy to introduce the intron 2 between the two fragments of PVY in inverted orientation under control of a CaMV35S promoter, to generate a double stranded RNA-encoding chimeric gene. To this end, the 0.6 kb HindIII S4 fragment from T-DNA vector pNS2 was replaced by the 0.77 kb HindIII fragment comprising the intron 2 described under paragraph 10. pNS2 comprised the gene components of expression vector N2 described on notebook page 151 (discussed in paragraph 5 above) cloned into T-DNA vector pART27 (see Exhibit 1, page 6 where it is indicated that the backbone vector is pART27, a T-DNA vector commonly used at CSIRO for introduction of chimeric DNAs into plant cells by Agrobacterium-mediated transformation). Notebook page 189 further describes the composition of the ligation mix and the restriction enzyme analysis to identify clones in which the S4 fragment had been replaced by the intron 2 fragment. A small table at the bottom of notebook page 189 indicates the predicted sizes of fragments generated by restriction enzyme digests for each of the two possible orientations of the inserted intron fragment (indicated as "sense intron" and "α-sense intron", the latter is shorthand for intron in antisense orientation).

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Page 5

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15. Exhibit 1 thus describes the successful construction of a chimeric DNA construct molecule comprising, in order:

- a) a promoter, operative in a eukaryotic (plant) cell (*CaMV35S*);
- b) a DNA region, which when transcribed, yields an RNA molecule with at least one RNA region with a nucleotide sequence comprising
  - i. a sense nucleotide sequence including at least 20 consecutive nucleotides (and also at least 50 or 100 nucleotides) having 100% sequence identity with at least 20 consecutive nucleotides (and also at least 50 or 100 nucleotides) of the nucleotide sequence of the nucleic acid of interest (*0.75 kb PVY region in sense orientation- the nucleic acid of interest is thus comprised in the genome of an infecting RNA virus in this embodiment*)); and
  - ii. an antisense nucleotide sequence including at least 20 consecutive nucleotides (and also at least 50 or 100 nucleotides) having 100% sequence identity with the complement of the at least 20 consecutive nucleotides (and also at least 50 or 100 nucleotides) of the sense nucleotide sequence (*0.75 kb PVY region in antisense orientation*);wherein the RNA is capable of forming an artificial hairpin RNA structure with a double stranded RNA stem by base-pairing between the regions with sense and antisense nucleotide sequences; and

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wherein the DNA region further comprises an intron (*intron 2*) (which is a heterologous intron with respect to the sense and antisense PVY sequences); and

- c) a DNA region involved in transcription termination and polyadenylation (*3' ocs region*).

16. The experiments recorded in Exhibit 1 were completed prior to December 23, 1997.

17. The experiments recorded in Exhibit 1 were performed in CSIRO Plant Industry laboratories in Canberra, Australia.

18. Exhibit 2 is a copy of 5 pages from a laboratory notebook (Notebook No 5) prepared by Ming-Bo Wang that recorded experiments performed by Ming-Bo Wang and/or under his supervision during the construction of a chimeric DNA encoding an RNA comprising sense and antisense nucleotide sequences targeted to a  $\beta$ -glucuronidase gene (GUS) in which the sense and antisense nucleotide sequences could basepair over about 558 bases, as described in the above mentioned US patent application in Example 1, Figure 1A (6) and Sequence Listing entry SEQ ID NO 1. Further recorded in Exhibit 2 are the introduction of the chimeric gene into a T-DNA vector and introduction of the resulting T-DNA vector into *Agrobacterium tumefaciens* comprising a disarmed helper Ti-plasmid. The introduction of the chimeric DNA into plant cells, and data showing reduction of target gene expression in the plant cells are recorded in Exhibits 4 and 5.

19. Exhibit 2, page 1, entitled "To make invert-repeat GUS construct for rice transformation" recorded the starting materials and ligation mixes set up to introduce a second copy of part of the GUS coding region into a vector pWUJGdT comprising a GUS coding region with a deletion of the internal 0.3kb EcoRV fragment.

20. Two cloning strategies were followed i.e.

- a. to introduce a HincII fragment from the GUS coding region derived from vector pWJKKGUS (indicated on top of the page as DNA 3) into EcoRI restricted vector pWUJGdT (indicated on top of the page as DNA 1 –

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treated to render the sticky ends of the restriction fragment blunt and further treated with alkaline phosphatase (AP) as indicated in the note at the top right of the page. This ligation mix is recorded as ligation 2.

- b. To introduce a *Sma*I-EcoRV fragment from the GUS coding region derived from vector pWJKBGUS (indicated on top of the page as DNA 4) into *Sma*I restricted vector pWUJGdT (indicated on top of the page as DNA 2 – treated with alkaline phosphatase (AP) as indicated in the note at the top right of the page). This ligation mix is recorded as ligation 4.

21. Exhibit 2, page 2 recorded the identification of candidate clones resulting from the cloning strategies, as well as identification of the orientation of the insert into the vector. At the bottom of the page, these two possible insert orientations into the chimeric DNA are schematically represented, resulting in two different vectors namely:

- a. candidate clone 2 (2) renamed pMBW234 where the chimeric gene comprised in order a promoter region from maize ubiquitin gene ("Ubi-P"); a 5' untranslated leader region from Johnsongrass mosaic virus ("JGMV"); a dysfunctional  $\beta$ -glucuronidase gene (GUSd) and the 5' end of the GUS coding region (GUS5') in the same orientation as the GUSd coding region, followed by transcription termination and polyadenylation region from *Agrobacterium* T-DNA gene *tml* (*tml*).
- b. candidate clone 2 (4) renamed pMBW233 where the chimeric gene comprised in order a promoter region from maize ubiquitin gene ("Ubi-P"); a 5' untranslated leader region from Johnsongrass mosaic virus ("JGMV"); a dysfunctional  $\beta$ -glucuronidase gene (GUSd) and the 5' end of the GUS coding region (GUS5') in the inverse orientation relative to the GUSd coding region, followed by transcription termination and polyadenylation region from *Agrobacterium* T-DNA gene *tml* (*tml*). Transcription of this chimeric gene yields an RNA molecule comprising an RNA region capable of forming an artificial hairpin RNA structure comprising two annealing RNA sequences, namely a sense RNA sequence identical to about 558 consecutive nucleotides of the GUS

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gene and an antisense RNA sequence identical to about 558 consecutive nucleotides of the complement of the GUS gene.

22. Exhibit 2, page 3 records the experiments performed to clone the chimeric genes described under paragraph 21, as NotI restriction fragments, into a T-DNA vector designated binary vector pWBVec4A. As indicated in the top right insert, "ligation 2" uses "DNA 1", i.e. NotI fragment from pMBW234 ; "ligation 3" uses "DNA 2" i.e. NotI fragment from pMBW233.

23. Exhibit 2, page 4 contains the continued recorded data from the experiments described under paragraph 22, including the restriction enzyme analysis, of candidate clones from ligations 2 and 3 mentioned on the previous page. The plasmid denoted pMBW237 refers to the chimeric gene in colony 2(8) [clone 8 of ligation mix 2, thus corresponding to a chimeric gene as in pMBW234], and the plasmid denoted pMBW239 refers to the chimeric gene in colony 3(8) [clone 8 of ligation mix 3, thus corresponding to a chimeric gene as in pMBW233].

24. At the bottom of Exhibit 2, page 4 it is indicated that T-DNA vectors 2(8) and 3(8) were used for triparental mating, referring to the introduction of the T-DNA vectors from the *E. coli* host into *Agrobacterium tumefaciens* comprising a disarmed helper Ti-plasmid, and Exhibit 2, page 5 records the verification of the *Agrobacterium* transconjugants as containing the respective T-DNA vectors.

25. The experiments recorded in Exhibit 2 were completed prior to December 23, 1997.

26. The experiments recorded in Exhibit 2 were performed in CSIRO Plant Industry laboratories in Canberra, Australia.

27. Exhibit 3 is a copy of a publication by Alan H. Christensen and Peter H. Quail, entitled "Ubiquitin promoter-based vectors for high-level expression of selectable and/or screenable marker genes in monocotyledonous plants", which was published in *Transgenic Research* Volume 5, 213-218 (May 1996). This publication describes the ubiquitin promoter region fragment used in the construction of the chimeric genes described in Exhibit 2, as explained under paragraph 21. As indicated in Figure 1, the Ubi promoter region included an intron (Ubi-1 intron) indicated in the Figure by an angled line) in the transcribed region. This publication is mentioned in the above mentioned patent application as a reference for the Ubiquitin promoter used in the chimeric constructs described in Example 1 of that patent

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application. At the time of the construction of the chimeric genes, described under paragraphs 20 and 21, we were aware of the presence of an intron in the transcribed DNA region under the control of the Ubi promoter region.

28. Exhibit 4 is a copy of 6 pages from a laboratory notebook (Notebook 96) prepared by Ming-Bo Wang that recorded experiments performed by Ming-Bo Wang and/or under his supervision to transform transgenic rice calli containing and expressing a recombinant GUS gene as a target gene of interest in a eukaryotic (plant) cell, with the T-DNA vectors described in Exhibit 2, and to analyse phenotypic expression of GUS in the supertransformed rice calli comprising the target gene and the chimeric DNAs. Data from these analyses correspond to the data recorded in the patent application as "Example 1".

29. Exhibit 4, page 1 indicates that calli were initiated from transgenic rice seeds 4R-V10-28 and 4R-V10-67, both expressing a recombinant GUS gene of interest integrated in the genome of the calli cells. This target gene is a transgene in the cells. The bottom half indicates that these calli were "supertransformed" using different Agrobacteria comprising the different T-DNA vectors including pMBW239 (experiment indicated as "7"). Note also the control experiments where an "empty" control T-DNA vector (pMBW223) was used (experiment 1) and where a conventional co-suppression construct (comprising a transcribed sense nucleotide sequence of the GUSd gene (pMBW225) was used for comparison of the level of phenotypic expression of the target gene (experiment 2).

30. Exhibit 4, page 2 recorded the data for Bialaphos resistant calli obtained with the different Agrobacteria described on the previous page (paragraph 24 above), indicating that such calli had been transformed by the T-DNA's including the chimeric DNA silencing constructs (or control constructs). The transgenic calli obtained by transformation of the initial V10-28 transgenic rice cells with pMBW239 are indicated as "7a" whereas the transgenic calli obtained by transformation of the initial V10-67 transgenic rice cells with pMBW239 are indicated as "7b".

31. Exhibit 4, page 2 at the bottom and continuing on page 3, recorded the data of an initial GUS staining experiment on parts of the transgenic calli that were obtained (V10-67 derived). In control experiment "1b" where the GUS transgenic calli were supertransformed by an "empty" T-DNA vector, all supertransformed calli strongly stained blue indicating strong GUS activity. On page 3 of Exhibit 4, the results of a similar GUS staining on calli obtained by supertransformation with pMBW239 indicating that the observed GUS staining was either weak

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("W") , negative ("-") or resulted in an isolated blue spot. These data indicated that the chimeric DNA from pMBW239 reduced the phenotypic expression of the GUS target gene in the rice cells.

32. Exhibit 4, pages 4 and 5 represent the recordation of these data for the individual calli. Exhibit 4, page 6 summarizes the data of an initial GUS staining experiment on parts of the obtained transgenic calli (V10-28 derived). While in series 1a, all calli transformed by the control T-DNA vector pMBW223 strongly stained blue indicating strong GUS activity, the transgenic calli obtained by transformation with pMBW239 (series 7a) all scored as "very weak" or "basically GUS negative" with the exception of only two calli. On the bottom of page 6, reference is made to (quantitative) GUS assays, the data of which are recorded in Notebook 5, starting on page 55, discussed hereinafter as Exhibit 5.

33. The experiments recorded in Exhibit 4 were completed prior to December 23, 1997.

34. The experiments recorded in Exhibit 4 were performed in CSIRO Plant Industry laboratories in Canberra, Australia.

35. Exhibit 5 is a copy of 17 pages from a laboratory notebook (Notebook 5) prepared by Ming Bo Wang that recorded quantitative GUS analysis experiments performed by Ming-Bo Wang and/or under his supervision, on the supertransformed rice calli described in Exhibit 4. Particular attention is drawn to the "raw data" concerning GUS analysis by kinetic value measurements on Exhibit 5, page 14 which are represented in Example 1, Table 2 of the above mentioned patent application. See e.g. data for Plate No 3, wells 1A to 2H which have in handwriting indications "7a1" to "7a14" referring to transgenic lines, which are identical to the entries in Table 2 of the patent application, column indicated by "Inverted repeat CoP(6)", row V10-28. Similarly, data for Plate No 3, wells 3A to 4D which have in handwriting indications "7b2" to "7b16" referring to transgenic lines are identical to the entries in Table 2 of the patent application, column indicated by "Inverted repeat CoP(6)", row V10-67.

36. The experiments recorded in Exhibit 5 were completed prior to December 23, 1997.

37. The experiments recorded in Exhibit 5 were performed in CSIRO Plant Industry laboratories in Canberra, Australia.

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38. The experiments recorded in Exhibit 2, 4 and 5 therefore describe successful completion prior to December 23, 1997 of the construction of a chimeric DNA molecule (**pMBW233/239 series**) comprising in order

- a. a promoter operative in a eukaryotic (plant) cell (**Ubi-P**);
- b. a DNA region, which when transcribed, yields an RNA molecule with at least one RNA region with a nucleotide sequence comprising
  - i. a sense nucleotide sequence including at least 20 consecutive nucleotides (and also at least 50 or 100 nucleotides) having 100 % sequence identity with at least 20 (and also at least 50 or 100 nucleotides) consecutive nucleotides of the nucleotide sequence of a nucleic acid of interest (**Gusd in sense orientation**) in a eukaryotic cell; and
  - ii. an antisense nucleotide sequence including at least 20 consecutive nucleotides (and also at least 50 or 100 nucleotides) having 100% sequence identity with the complement of the at least 20 consecutive nucleotides (and also at least 50 or 100 nucleotides) of the sense nucleotide sequence (**Gus5' in antisense orientation**);

wherein the RNA is capable of forming an artificial hairpin RNA structure with a double stranded RNA stem by base-pairing between the regions with sense and antisense nucleotide sequence wherein the DNA region further comprises an intron (**Ubi intron**) (which is heterologous to the sense GUS sequence); and

- c. a DNA region involved in transcription termination and polyadenylation (**tml'**).

39. The experiments recorded in Exhibit 2, 4 and 5 also describe successful completion prior to December 23, 1997 of a method for reducing the phenotypic expression of a nucleic acid of interest which is normally capable of being expressed (**GUS gene**) in a eukaryotic cell (a plant cell, **rice**) comprising the step of introducing into the eukaryotic cell (plant cell) a chimeric DNA (**pMBW233/239 series**) as described in paragraph 24.

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40. The statements made in this declaration are made on the personal knowledge or on the information and belief of the declarants. We hereby declare that all statements made herein of personal knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.


Date: \_\_\_\_\_

\_\_\_\_\_  
Peter Michael Waterhouse,

Date: \_\_\_\_\_

\_\_\_\_\_  
Ming-Bo Wang

Date: 2 May, 2008

  
\_\_\_\_\_  
Michael Wayne Graham

Date: \_\_\_\_\_

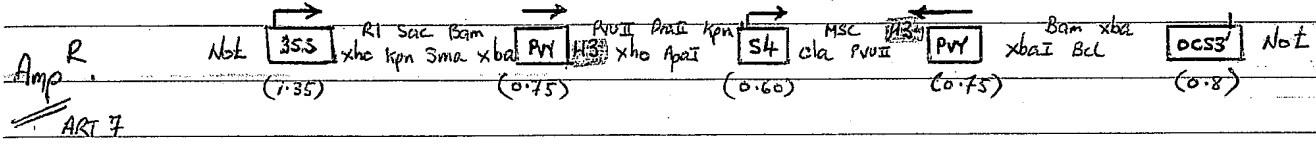
\_\_\_\_\_  
Neil A. Smith



Experiment: ..... WEDNESDAY ..... Date: .....

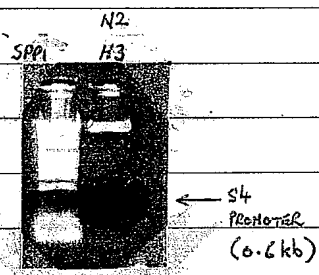
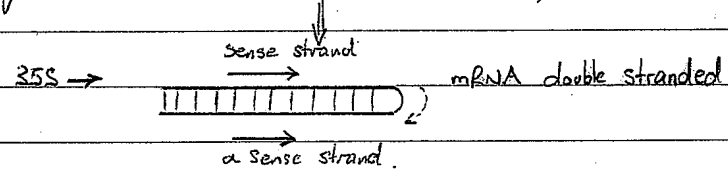
CREATION OF PVY mRNA HAIRPIN ("DOUBLE STRANDED")

USING: N2 p 82 + 96 \*



\* Cut 10 $\mu$ l of N2 with H3 o/n 37 $^{\circ}$ C

Will religate in 100 $\mu$ l to delete S4 promoter

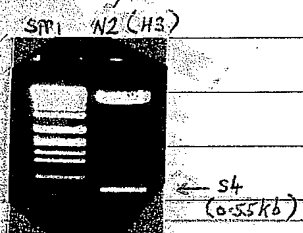


LIGATION

VECTOR: 20 $\mu$ l digest RT.  
BUFFER: 2.0 $\mu$ l  
H<sub>2</sub>O: 62.0 $\mu$ l  
LIGASE: 3.0 $\mu$ l  
ATP(5mM): 5.0 $\mu$ l No colonies Rpt'd  
TOTAL: 100.0 $\mu$ l

Rpt. Cut 10 $\mu$ l N2 with H3 for 2 hours. (200 $\mu$ l Reaction)  
Resuspended in 10 $\mu$ l dH<sub>2</sub>O. Ran lnd.

LIGATION: Rpt  
Cut Plasmid: 9.0 $\mu$ l  
Buffer: 2.0 $\mu$ l  
dH<sub>2</sub>O: 7.0 $\mu$ l  
Ligase: 1.0 $\mu$ l  
ATP(5mM): 1.0 $\mu$ l  
TOTAL: 20.0 $\mu$ l



\* No clones - All still have insert!  
Will repeat by cutting band from TAE Agarose & ligating.

Signature of Researcher	Cont. PAGE 154	Zapped $\rightarrow$ 8452 cells
Signature of Supervisor	Def. Gull	2nd $\rightarrow$ JM109 cells
		Date

Experiment: .....

WEDNESDAY

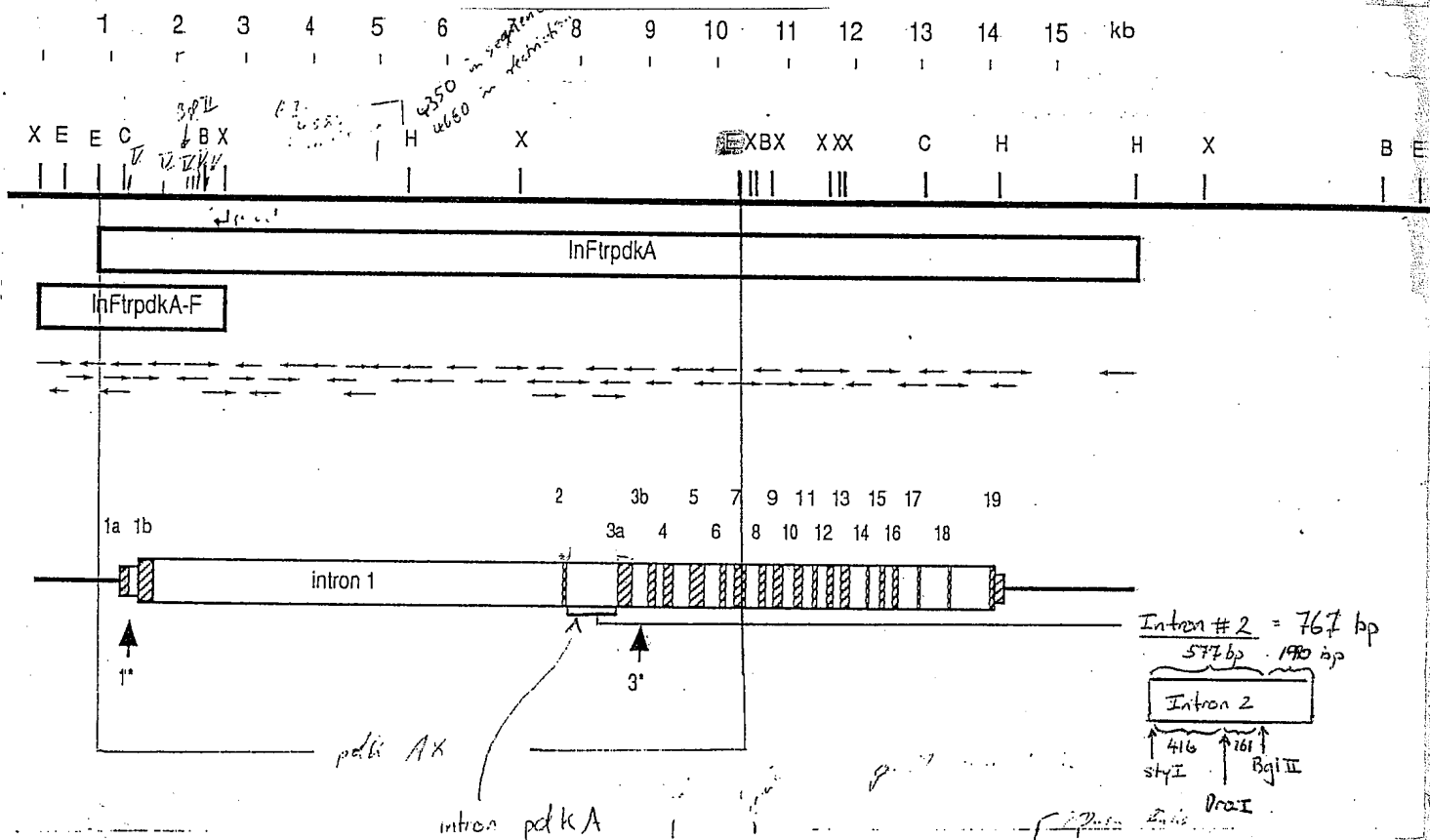
Date: .....

PREPARATION OF INTRON (2) FROM FLAVARIA travenaria

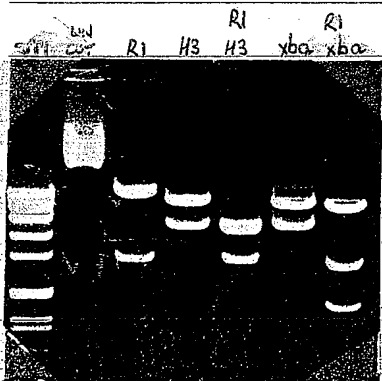
Plasmid provided by Bill Taylor

pdkA = *Flavaria travenaria*pdkB = *Flavaria brownii*

pdk1 - 13.75 kb plasmid

Intron is in pBluescript (ks) as an ~~ECO~~ ~~RI~~ fragment (10.5 kb) - T3-T7 orientat<sup>n</sup>

Set up Quagen prep + did diagnostic cuts



✓ R1 - 10.5 kb (insert) + 3.0 kb (Vector)

✓ H3 - 5.5 kb (5' end intron) + 7.5 (Rest)

✓ R1/H3 " " 4.5 kb (3' end) + 3.0 kb (Vector)

✓ xba ~ 4-4.5 kb (centre)

R1/xba -

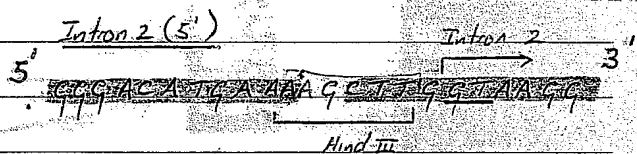
DNA STD (ng) Quagen (ul)  
20 150 100 50 0.1 0.25 0.5

= 200 ng / ul

W. Taylor

potentieller Translationsstart		
pdka	- ATTGAATAAAAAA---CATTTGATTITGACITTTGGTTTGGATTGATTATG	- 6624
pdkB	- ATTGAAGAAAAAACCATTTGATTITGACITTTGGTTTGGATTGATATG	- 2868
pdka	- CAGAGGGTTTTCACITTTGGTAAAGGAAGTGAAGGCAACAGGACAT	- 6674
pdkB	- CAGAGGGTTTTCACITTTGGTAAAGGAACAGTGAAGGCAACAGGACAT	- 2918
pdka	- GAAATCCTTGGTAAAGGAATAATTATTTTCCTT---TTTCCTTTTAGTATA	- 6722
pdkB	- GAAATCCTTGGTAAAGGAATCATTATTTTCCTTTCCTTTAATTATAAGT	- 2968
pdka	- AAATA---GTTAAGTGATGTTAATTAGTATGATTATAATAATATAGTTG	- 6768
pdkB	- AAGTAATGTGTTAAGTAATGTTAATTAGCATGATTATATAATATGATTG	- 3018
pdka	- TTATAATTGTGAAAAATAATTTATAAATATATTTGTTTACATAAACAACA	- 6818
pdkB	- TTATAATTGTGAAAAAG-AAITTTATAAATATATGTTTAGATAAACAAC	- 3067
pdka	- TAGTAATGTAAAAAATATGACAGTGATGTGAAGACGAAGAAGATAAA	- 6868
pdkB	- TACTAAGGTAAAT---TTATAACAGTGATGTGAATCCTAAGAAGATAAA	- 3115
pdka	- AG-TTGAGAGTAAGTATATTATTTTAAATGAATTGATCGAACATGTAAG	- 6917
pdkB	- AAATTAAAGTAAATATATT---TTTAATAAATTTGA---ACATATAAG	- 3158
pdka	- ATGA-TA-TACTAGCATTAAATTTGTTTTAATCATAATAGTAAT----	- 6960
pdkB	- ATGACTAATAAATACATTAAATTTGTTTAAATCATAATAGTAATAGTAA	- 3208
pdka	- TCTAGCTGGTTTGATGAATTAATATCAATGATAAAATCTATAGTAAA	- 7009
pdkB	- TTCTAGTTGGTTTGATGAATTAATA-CAATGATAAAGTACTATAGTAAA	- 3257
pdka	- AATAAGAATAAATAAATTAATAATATTTTATGATTAAATAGTTTAT	- 7059
pdkB	- AATAAGAATAAGTAAATTAATAATATT---ATGATTAGTATTAT	- 3302
pdka	- TATATAATTAAATATCTATACCATTACTAAATATTTAGTTTAAAGTTA	- 7109
pdkB	- TAGATAATTAAATATCTATACCATTACTAAATATTTAGTATAAAGTTA	- 3352
pdka	- ATAAATATTTTGTGTAGAAATCCCAATCTGCTTGTA---ATTATCAATAAA	- 7157
pdkB	- ATAAATATTTTGTGTGAAATCCCAATCTGCTTGTAATTTATCAATAAA	- 3402
pdka	- CAAAATATTAAATAACAAGCTAAAGTAACAAATAATATCAAACTAATAGA	- 7207
pdkB	- CAAAATTTTAAATAACAAGCTAAAGTAACAAATAATATCAAACTAATAAA	- 3452
pdka	- AACAGTAATCTAATG-----TAACAAAAC-----	- 7231
pdkB	- AACAGTAATCTAATGCTAATATAACAAAACGCAACGCTATCAATTTTAT	- 3502
pdka	- ATAATCT-----AATGCT---	- 7244
pdkB	- ATAATATTATTTTAAATTAACATCTTATTATTCTAATAATACCTTGT	- 3552
pdka	- AGTATTACCAATTATCACCATAAATAATTATGATGAACACTATGTCAA	- 3602
pdka	- AATATAAC-----AAGCGCAAGA-----TCTATCA	- 7270
pdkB	- AATATAAATAAATAAATAAATGCAATAGAAATTTAAACATTCATCC	- 3652
pdka	- TTTT-----ATAT---	- 7278
pdkB	- TTTTITTTACTAATGATTTTGGAAATCACTAATGGATATGAATCCTTATTA	- 3702
pdka	- TTAATGAATTTAAAGCAACTCATAGTGAGCGTTTGGTTTGAAGGAATGGA	- 3752
pdka	- AGTATTATTTTCAA-----	- 7292
pdkB	- ATGGAATGGAACGGAATGAGAACTAGTATAATTTTCAAGTAAATAT	- 3802
pdka	- TCAAC---A-----	- 7298
pdkB	- ATTTGAGTGGTTAATGAATGTAAACCCAAGGGAATGTTTCACTTCATA	- 3852
pdka	- TTC-----TTATTAATTT---	- 7311
pdkB	- TTCCCTTGGTTTGGGATTTTGGATTTCACCTAGGGAGGGAATTTGAA	- 3902

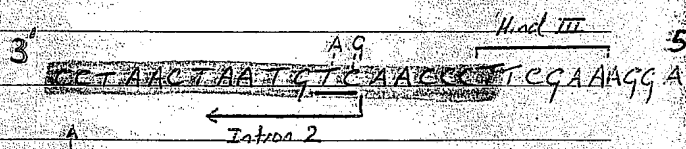
Designed primers



23 mer - 11 GC

12 TA oligo 193 ng/ul

Intron 2 (3') LABELED Intron 2 (3')



Complementary strand

28 mer

oligo = 245 ng/ul

P.T.O

Nef/Satt

Date / /

Date / /

MCTab

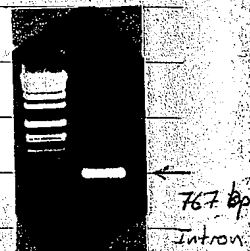
Experiment:

TUESDAY Date

## AMPLIFICATION OF Intron 2 from Pdk1

## PCR REACTION - Set up 2

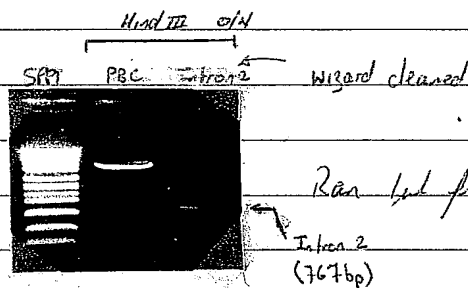
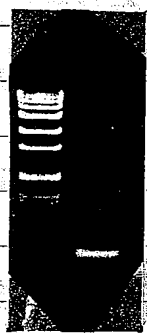
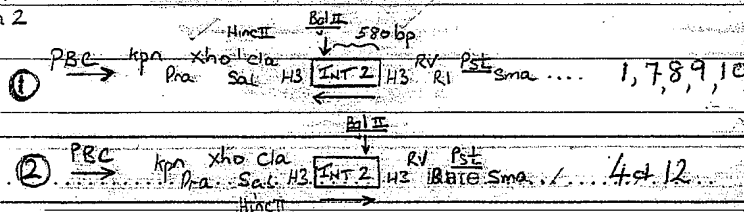
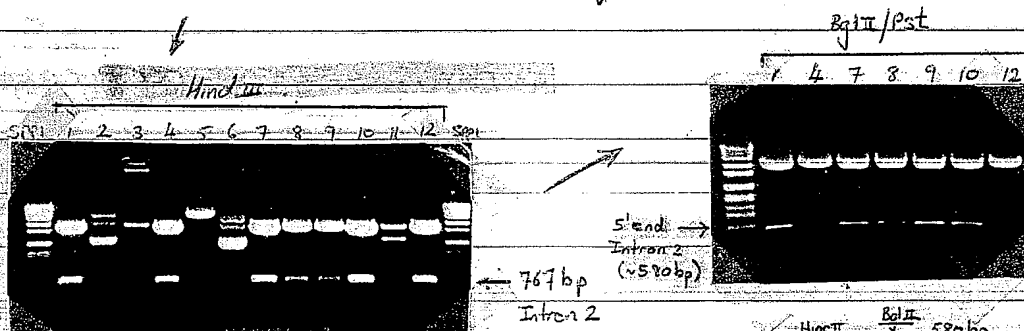
DNA (20ng)	1.0 $\mu$ l	[ 95°C 2mins ] x 1
Amplitag BUFFER (x10)	1.0 $\mu$ l	[ 95°C 20sec, 56°C 30 sec, 72°C 1min ] x 30
mgcl <sub>2</sub> (25mM)	1.0 $\mu$ l	[ 72°C 5 mins ] x 1
P.187 { 5' PRIMER (20ng)	1.0 $\mu$ l	* Did 2 identical reactions (10 $\mu$ l each)
3' PRIMER (20ng)	1.0 $\mu$ l	
dH <sub>2</sub> O	3.8 $\mu$ l	Ran 2.5 $\mu$ l of one reaction
2.5 mM dNTP <sup>3</sup>	1.0 $\mu$ l	* Pooled remainder, & prep etc
Amplitag Polym	0.2 $\mu$ l	↓ 2 $\mu$ l from 10 $\mu$ l
TOTAL	10.0 $\mu$ l	



## LIGATION Intron 2 → PBC

Cut rest (8  $\mu$ l) in 200  $\mu$ l  
Reaction with Hind III

VACUOL (PBC-H3)	1.0 $\mu$ l	✓
INSER (Intron 2 PCR) H3	12.0 $\mu$ l	✓
BUFFER	2.0 $\mu$ l	✓
H <sub>2</sub> O	3.0 $\mu$ l	✓
LIGASE	1.0 $\mu$ l	
ATP (5mM)	1.0 $\mu$ l	✓
TOTAL	20.0 $\mu$ l	@ 25°C

chl<sup>R</sup> B/W selection Cut with Bgl II / Pst → 577 bp OR 190 bp

Na/Smith

Signature of Researcher

Signature of Supervisor

Hinc II (blunt)

Date

Na/Smith

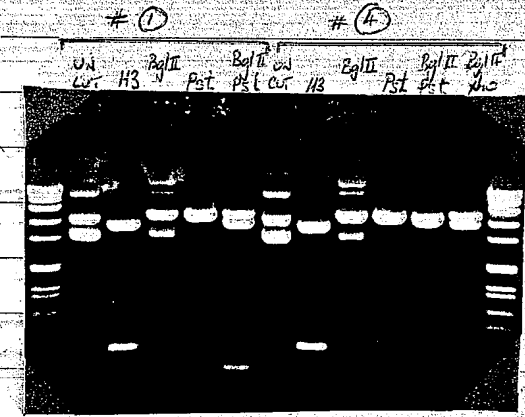
Na/Smith

Experiment:

Declaration 1  
Exhibit 1 Page 5  
US 09/287,632

Date / /

INTRON 2 IN PBC



#1 - In ori ~~1~~ p 188  
#4 - In ori 2 p 188

NS: BglII has only partially cut

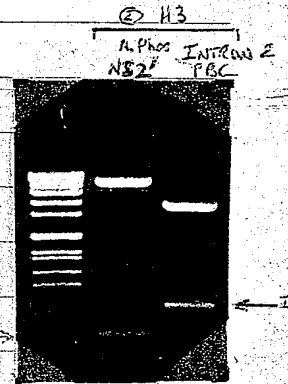
\* All cuts are correct \*

INTRON 2 → pNS2 (35S/PVY / PVY/ocs 3')  
(cut with H3)

Amp<sup>R</sup>

SET UP 2 LIGATIONS (1) & (2)

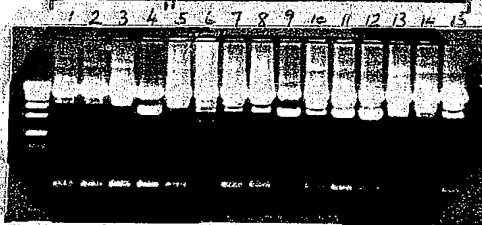
VECTOR (NS2-H3 A Phosph <sup>d</sup> )	① 5.0 μl	② 1.0 μl
INSERT (INTRON 2/PBC H3)	✓ 5.0 μl	✓ 5.0 μl
BUFFER	✓ 2.0 μl	✓ 2.0 μl
H <sub>2</sub> O	✓ 6.0 μl	✓ 10.0 μl
LIGASE	✓ 1.0 μl	✓ 1.0 μl
ATP (5mM)	✓ 1.0 μl	✓ 1.0 μl
TOTAL	20.0 μl	20.0 μl



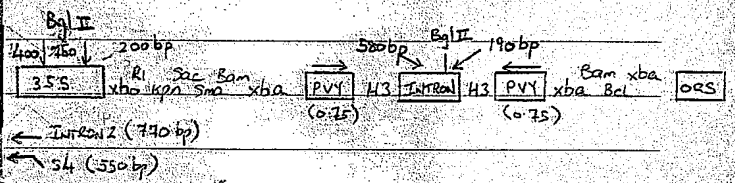
R.T. ligation

NS2 cut looks partial

SINGLE COLONIES Pools x 6



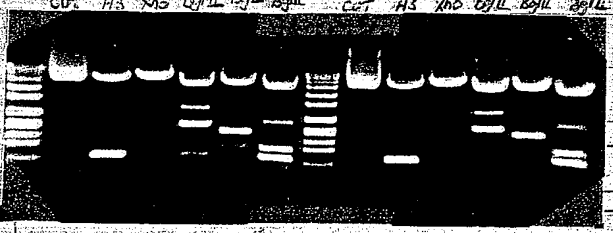
SINGLE COLONIES Pools x 6



142 (H3 cut 5')

① xho H3  
cut H3 xho BglII BglII

Sense INTRON  
① H3 → 770 bp (INTRON)  
② xho → Linearize



✓ BglII → 1.530 kb (35S 3' / PVY / INT 5') + 750 (from 35S) + 750 (35S 3')  
✓ xho/BglII → 1.33 (PVY / INT 5') + 750 (35S 3') + 950 (35S 3')  
✓ H3/BglII → 950 bp (35S 3' / PVY)  
✓ 580 (INT 5')  
✓ 190 (INT 3')

Signature of Supervisor

CONTINUED P. 192

All correct  
in Hand

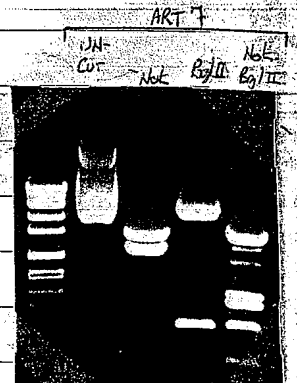
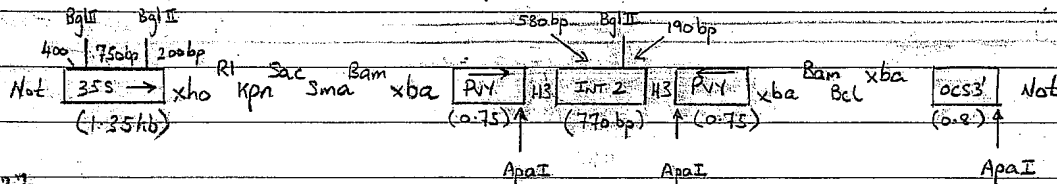
DATE

WATER

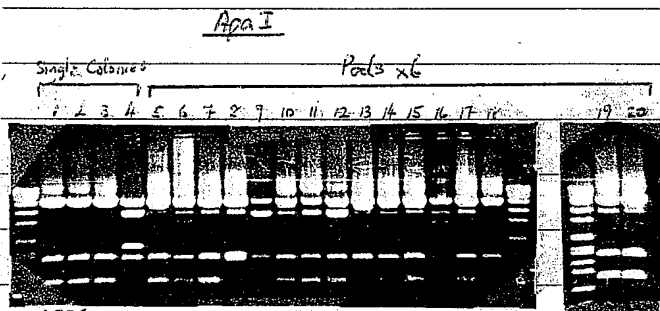
Experiment:

THURSDAY Date

Intron 2 → PVS 2 (PVY HAIRPIN) CONT' P189

RB  
IN ART 27CONFIRMS 2 BglII  
SITES IN 35S

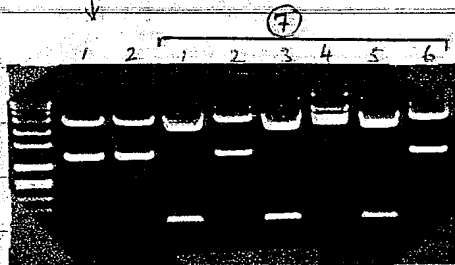
CUT Mini Preps Page 189 (20) with ApaI to confirm single inserts



Expect

770 bp (Intron) +  
~1.5 kb (PVY/ocs3') (confirms with cuts p. 189)

✓ Appear to be many with single Intron 2 inserts

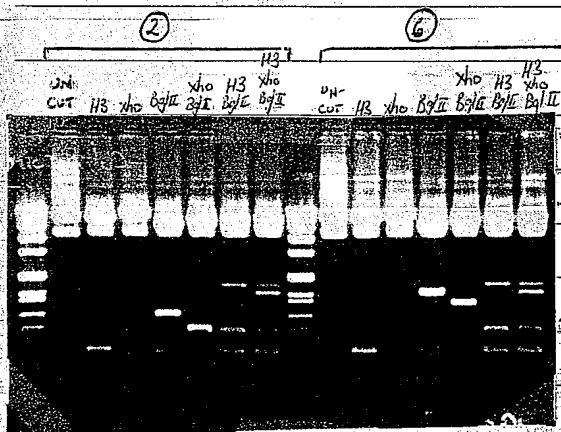
Group single Colonies from #7  
+ cut with xbaI

Expect

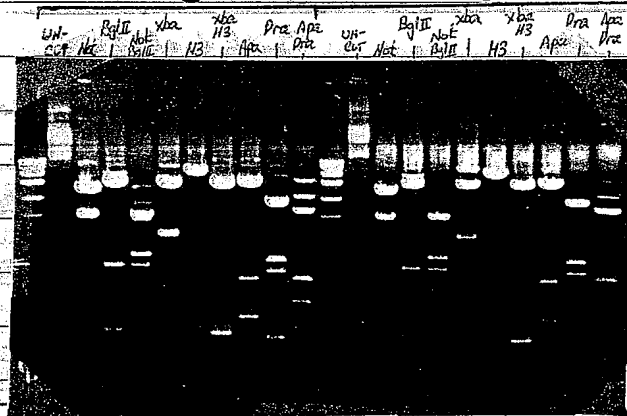
XbaI → ~2.3 kb (PVY/INT/PVY)

Again original 142 (p. 189)

+ 246 (from pool 7) appear to have intron insert

PVY  
deletion?  
due to Hairpin  
structure?FROM original  
single

⑥ (FROM Pool 7)

ART  
27INTRON IN  
Signature of ResearcherINTRON IN  
SENSE ORI

Signature of Supervisor

SET UP DIAGNOSIS OF ② + ⑥

Date

Date

UCI

Nail Smith



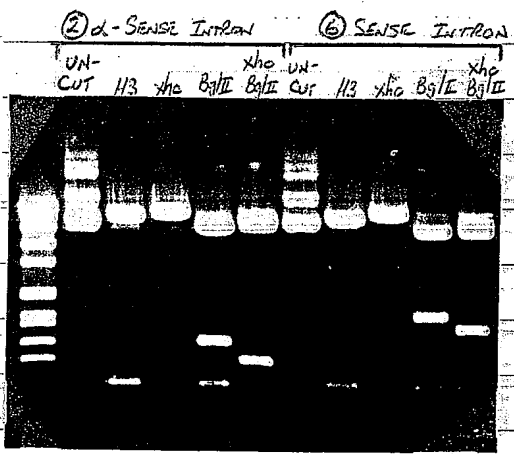
Experiment: ..... MONDAY, Date .....

DIAGNOSTIC CUTS

Not  
BgII BglII



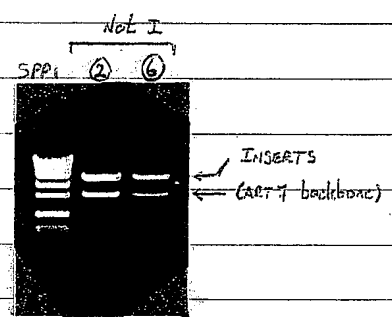
2-BglII  
355



✓ All Cuts are correct

NOTED: (2)  $\alpha$  Sense Intron as pNS 9  
(6) SENSE Intron as pNS 10

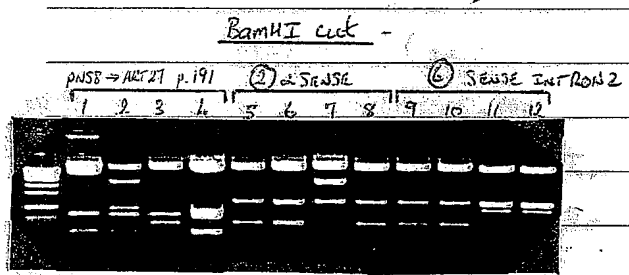
inserts



LIGATIONS For (2) + (6)  $\rightarrow$  ART 27

- ✓ VECTOR (ART 27 p.106)  $\frac{1}{20}$  : 1.0  $\mu$ l
- ✓ INSERTS ((2) or (6) Not I) : 4.0  $\mu$ l
- ✓ BUFFER : 2.0  $\mu$ l @ R.T o/h
- ✓ H<sub>2</sub>O : 11.0  $\mu$ l
- ✓ LIGASE : 1.0  $\mu$ l B/W Spec<sup>R</sup>.
- ✓ ATP (5mM) : 1.0  $\mu$ l

insert

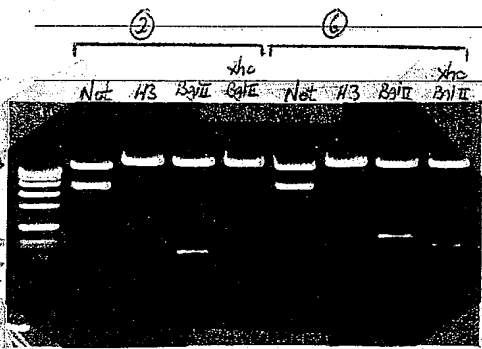


FOR (2) + (6)

✓ 355 at RB = 1.3 kb (ass's + sense apt II)  $\rightarrow$  2.20 kb (PVI / INT)

\* check # (8) + (9) FOR FURTHER CUTS

✓ MORE CUTS TO CONFIRM



\* SET UP (2) + (6) FOR TRI-PARENTAL  $\rightarrow$  LGA 4404

Expected

NS8			NS9			NS10			NotI		4.75 (INSAR)	
NotI	XhoI	NotI	XhoI	NotI	XhoI	NotI	XhoI	NotI	XhoI	1.55 + 1.05 kb		
									NS8	XhoI	4.40	
									NS9	NotI	2.30	
									NS10	XhoI	4.40	
									NS10	NotI	2.30	

Signature of Researcher

Signature of Supervisor

\* All correct \*

Date

W. J. ...  
Not/Smith

W. J. ...

Project \_\_\_\_\_ (code) \_\_\_\_\_ Book No: \_\_\_\_\_ Cont'd from page \_\_\_\_\_

Experiment Title To make invert-repeat GUS construct for rice transformat

① } pWUJadT 2ul } pWUJadT 1ul V: Purify ① with  
10X EcoRI 5 } 10X buffer 2 phenol/chloroform ex. 5  
EcoRI 2.0 } SmaI 1.5 treat with label kit  
H<sub>2</sub>O 41ul } H<sub>2</sub>O 15.5 to blunt. Then purify  
with phenol/chloroform ages  
followed by AP treatment

③ } pWTKK-GUS 5ul } pWTKK-GUS 5ul Δ: Treat ② with AP.  
10X buffer 5 } 10X buffer 5  
HincII 2 } SmaI\* 2 \* SmaI added first.  
H<sub>2</sub>O 38 } EcoRV 2  
H<sub>2</sub>O 36



Run 4.5ul of each reaction (during digest  
or AP treatment)

Finally purify with phenol/chloroform.  
20ul DNA in H<sub>2</sub>O.

Ligation:

1. { DNA 1 2ul } 2. { DNA 1 2ul } 3. { DNA 2 2.0 } 4. { DNA 2 2.0 }  
10X lig 2 } DNA 3 3.0ul } 10X lig 2 } DNA 4. 3.0 }  
ATP 1 } 10X lig 2 } ATP 1 } 10X lig 2 }  
ligase 0.5 } ATP 1 } ligase 0.5 } ATP 1 }  
H<sub>2</sub>O 14.5 } ligase 0.5 } H<sub>2</sub>O 14.5 } ligase 0.5 }  
H<sub>2</sub>O 11.5 } H<sub>2</sub>O 11.5 }

4°C,

Cont'd on page

Recorded by MING-BO WANG  
Print Name \_\_\_\_\_ Date \_\_\_\_\_

Supervisor  
e \_\_\_\_\_

Read and Understood by  
Print Name \_\_\_\_\_ Date \_\_\_\_\_

Signed Ming-Bo Wang

Initialled

Signed \_\_\_\_\_



Project

(code)

Book No:

Cont'd from page

Experiment Title



Analyse colonies of lig 2 and 4 on page 25

37 colonies for lig 2

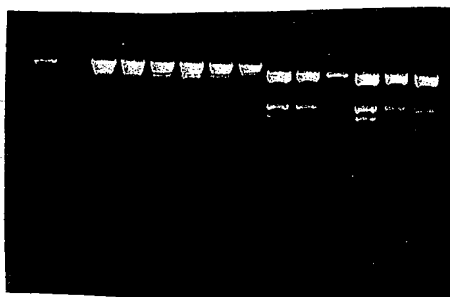
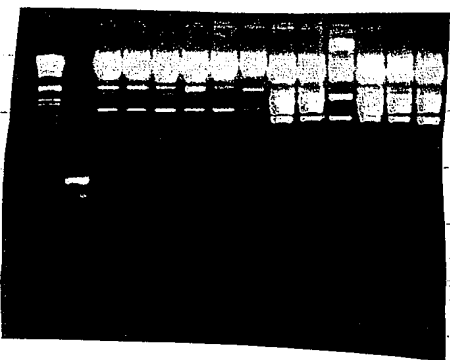
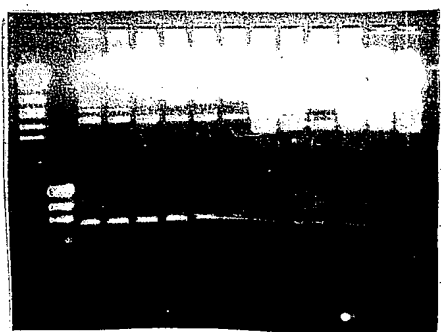
35 " " for lig 4

pool the 72 colonies into 12 tubes. 60 µl DNA in TC

Digestion:

{	1, 2, 3, 4, 5, 6	3 µl
	10X buffer	2
	SnaBI	0.5
	SacI	0.5
	H <sub>2</sub> O	14 µl

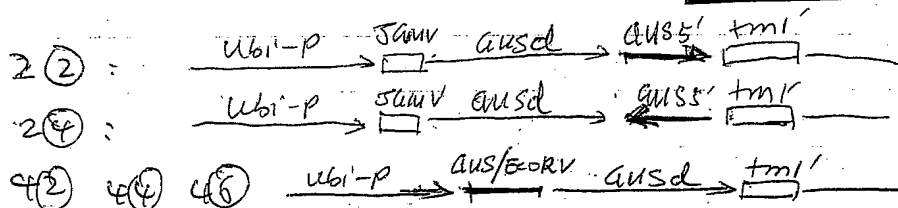
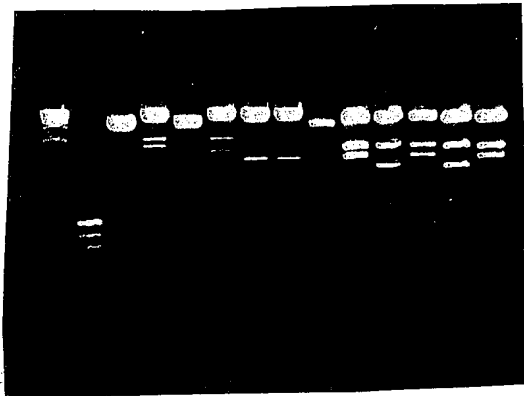
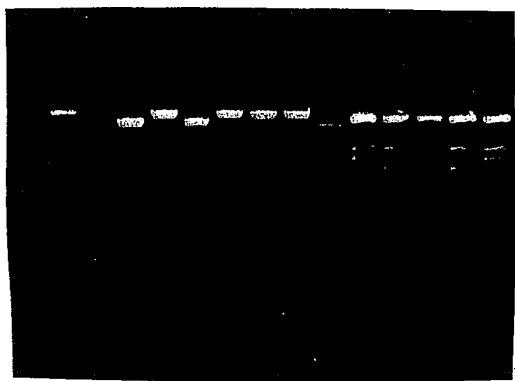
{	7, 8, 9, 10, 11, 12	
	10X EWR1	
	NcoI	0
	EcoRI	0
	H <sub>2</sub> O	14 µl



Prepare DNA from separate colonies in pool 1 and pool 7.

60 µl DNA in TCR.

Digest as above.



pmBW234

pmBW233

orientation not checked

Cont'd on page pmBW

Recorded by MING-BO WANG Date

Supervisor Date

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Signed Ming Bo Wang

Initialled

Signed MC TAG

ect

Experiment Title

Put pMBW232 and pMBW233 to binary pMBVecta

1. pMBW232 2.5  
10x buffer 3 5  
NotI 2  
H<sub>2</sub>O 40.5

2. pMBW233 2.5  
10x buffer 3 5  
NotI 2  
H<sub>2</sub>O 40.5

Ligations:  
1. DNA1 ( ) 1.5  
10x lig 2  
ATP 1  
ligase 0.5  
H<sub>2</sub>O 14.5

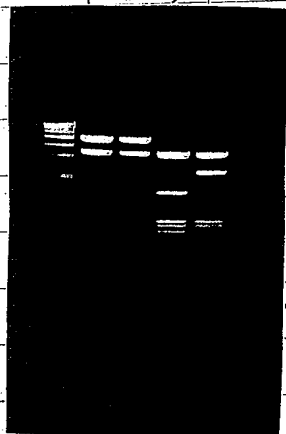
2. pNA ( ) 1.5  
DNA1 ( ) 3  
10x lig 2  
ATP 0.5  
ligase 0.5  
H<sub>2</sub>O 12

3. DNA1 ( ) 1.5  
DNA2C ( ) 3  
10x lig 2  
ATP 0.5  
ligase 0.5  
H<sub>2</sub>O 12

To further check:

3. pMBW232 1  
10x buffer 4 2  
NcoI 0.5  
HincII 0.5  
H<sub>2</sub>O 16

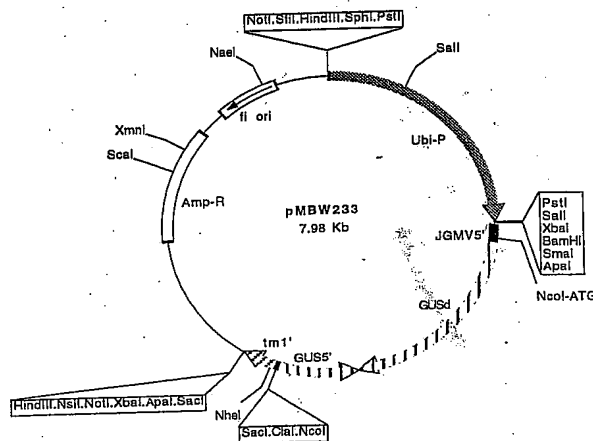
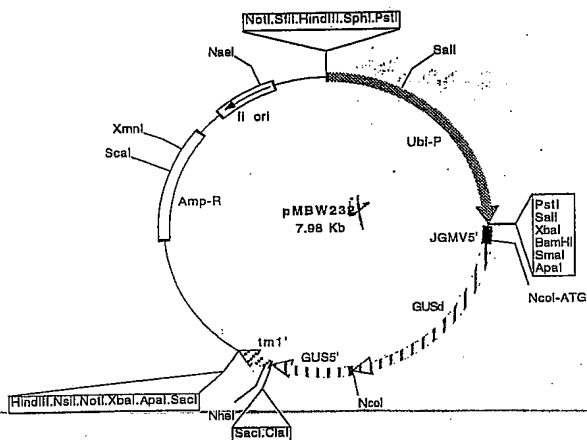
4. pMBW233 1  
10x buffer 4 2  
NcoI 0.5  
HincII 0.5  
H<sub>2</sub>O 16



Purify 1 and 2, 20 μl DNA in H<sub>2</sub>O.  
Run 2 μl each together with 3 and 4.

GUSd + GUS 5' (NcoI/HincII)  
GUSd  
ubi-P  
ubi-intron  
tm1' + GUS 5' (NcoI/HincII part.)  
→ tm1'

\* Both clones are correct.



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Project

(code)

Book No:

Cont'd from page

Experiment Title

Analyse colonies from lig 2 and 3 of

30 µg DNA is TCR.

Digestion:

DNA 1.5 µl

20 20 20 25 20 = pMBW236

10x buffer 2

24 27 28 29 = pMBW237

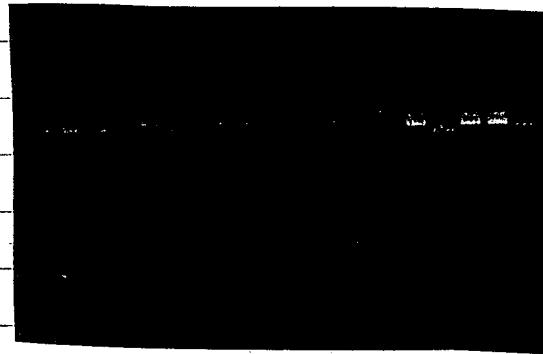
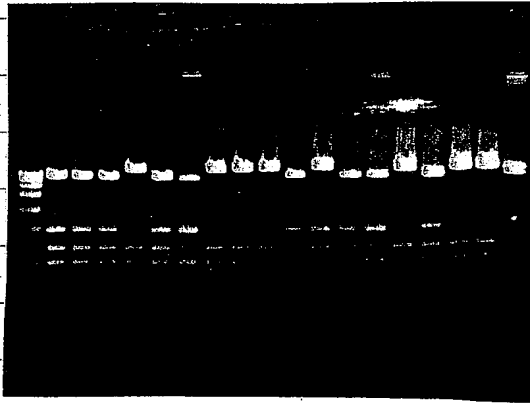
PstI 0.5

30 33 34 36 = pMBW238

H<sub>2</sub>O 16 µl

35 37 38 = pMBW239

32 and 39 did not look right.



Further check.

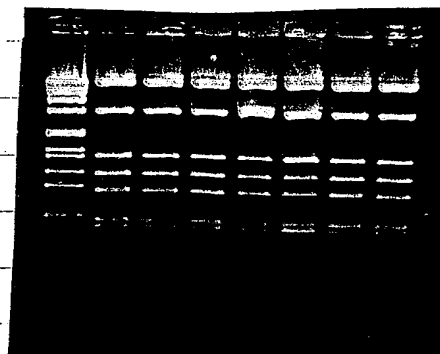
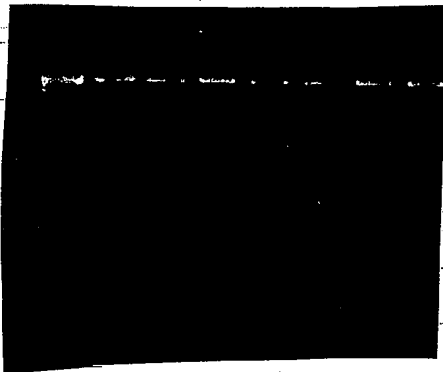
20 24 28 32 34 35 38 1.5 µl

10x buffer 2

NotI 0.5

~~PstI~~ 0.5

SalI

H<sub>2</sub>O 15.5

5' - GMS - GMS - tml  
- ubi - p 3' part  
- LB + tml + bar 3' half  
- RB  
- 355 - bar 5' half  
- RB or ubi - p 5' ?

28 and 38 were used for diploidal mating.

Cont'd on page

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Experiment Title

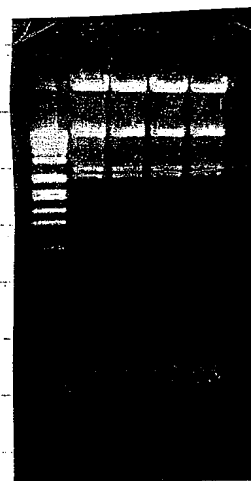
Analyse Agrobacterium disconjugants

1, 2 : AGLI = pmBW237 (28)

3, 4 : AGLI = pmBW239 (38)

Digestion:

DNA 5 µl  
10X buffer 3 2  
PstI 0.5  
H<sub>2</sub>O 12.5



check the TGMV vectors for KpnI

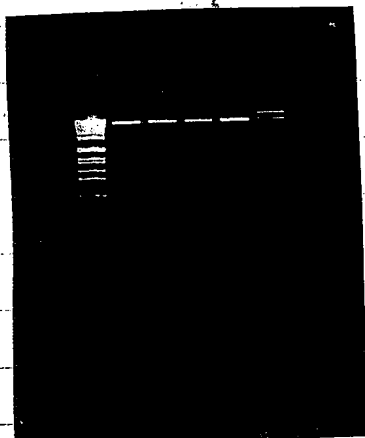
① pWUSJT (N=2) ② pWUSJCT (N=10) ③ pWUSJCT (18) ④ pWUSJBCT (22) ⑤ pWUSJBCT (16)

10X buffer 1

KpnI

H<sub>2</sub>O

2  
0.5  
16.5



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Signed .....

## TECHNICAL NOTE

# Ubiquitin promoter-based vectors for high-level expression of selectable and/or screenable marker genes in monocotyledonous plants

ALAN H. CHRISTENSEN<sup>‡</sup> and PETER H. QUAIL\*

Dept. of Plant Biology, University of California, Berkeley, CA 94720, and USDA/ARS Plant Gene Expression Center, 800 Buchanan St., Albany, CA 94710, USA (Fax: 1 510-559-5678)

Received 7 July 1995; revised 21 August 1995; accepted 24 August 1995

A set of plasmids has been constructed utilizing the promoter, 5' untranslated exon, and first intron of the maize ubiquitin (*Ubi-1*) gene to drive expression of protein coding sequences of choice. Plasmids containing chimaeric genes for ubiquitin-luciferase (*Ubi-Luc*), ubiquitin- $\beta$ -glucuronidase (*Ubi-GUS*), and ubiquitin-phosphinothricin acetyl transferase (*Ubi-bar*) have been generated, as well as a construct containing chimaeric genes for both *Ubi-GUS* and *Ubi-bar* in a single plasmid. Another construct was generated to allow cloning of protein coding sequences of choice on *Bam*HI and *Bam*HI-compatible restriction fragments downstream of the *Ubi-1* gene fragment. Because the *Ubi-1* promoter has been shown to be highly active in monocots, these constructs may be useful for generating high-level gene expression of selectable markers to facilitate efficient transformation of monocots, to drive expression of reference reporter genes in studies of gene expression, and to provide expression of biotechnologically important protein products in transgenic plants.

**Keywords:** gene expression; transgenic monocots; ubiquitin

## Introduction

The general availability of strong promoters active in all or most cell types of monocotyledonous plants would be useful in a variety of applications in gene transfer studies with this plant group (McElroy and Brettell, 1994). Although the widely-used cauliflower mosaic virus (CaMV) 35S promoter is active in monocot cells, its relative strength is substantially less than in dicot cells, and it is inactive in some cell types, e.g. pollen (Bruce *et al.*, 1989; Christensen *et al.*, 1992; McElroy and Brettell, 1994). The maize *Adh1* promoter has also been used in monocot transformation studies (Fromm *et al.*, 1990), but its activity appears to be restricted to root and shoot meristems, endosperm, and pollen (Kyojuka *et al.*, 1991). Because of their expected involvement in fundamental processes in all cell types, the genes for rice actin (*Act-1*) (McElroy *et al.*, 1990) and maize ubiquitin (*Ubi-1*) (Christensen *et al.*, 1992) have been investigated as

potentially useful alternatives to the CaMV 35S and *Adh1* sequences. Both of these monocot promoters have been shown to be significantly more active than the CaMV 35S promoter in monocot cells (Bruce *et al.*, 1989; McElroy *et al.*, 1990; Christensen *et al.*, 1992; Cornejo *et al.*, 1993; Gallo-Meagher and Irvine, 1993; McElroy and Brettell, 1994) with the *Ubi-1* promoter being somewhat stronger than the *Act-1* promoter where compared directly (Cornejo *et al.*, 1993; Gallo-Meagher and Irvine, 1993; Schledzewski and Mendel, 1994; Wilmink *et al.*, 1995).

Since our initial reports on the use of maize *Ubi-1* promoter constructs in transient (Christensen *et al.*, 1992) and stable (Toki *et al.*, 1992; Uchimiya *et al.*, 1993) cereal transformation studies, we have distributed to a large number of researchers a variety of constructs with the *Ubi-1* promoter fused to a spectrum of selectable and scorable markers. Certain of these constructs or their derivatives have been used successfully in transforming a number of different monocot species (Wilmink *et al.*, 1995), including several cereals (McElroy and Brettell, 1994) and *Lemna* (Rolfe and Tobin, 1991), with reports of transgenic plants having been generated for rice (Cornejo *et al.*, 1993), wheat (Weeks *et al.*, 1993), and

\*To whom correspondence should be addressed.

<sup>‡</sup>Present address: Dept. of Biology, George Mason University, Fairfax, VA 22030, USA

barley (Wan and Lemaux, 1994). This report presents the structural details of the complete set of these constructs.

## Materials and methods

The cloning and sequencing of the maize ubiquitin gene and its promoter have been reported previously (Christensen *et al.*, 1992). All DNA cloning and manipulations reported here were performed according to standard protocols (Ausubel *et al.*, 1989). Restriction endonuclease digestions were carried out according to manufacturers' recommendations. GeneClean (BIO 101, La Jolla, CA, USA) was used to isolate specific restriction fragments from agarose gels. Recovery of DNA fragments was quantified by comparison of ethidium bromide fluorescence of an aliquot of the fragment with known masses of DNA on agarose gels. The *Hind*III linker (5' CAAGCTTG 3') used in the construction of pAHC27 was obtained from New England Biolabs. DNA ligations and subsequent transformations into competent *Escherichia coli* strain XL1-Blue or HB101 cells and plasmid DNA preparations were carried out using standard protocols (Ausubel *et al.*, 1989). Analysis of DNA sequences was performed using the UWGCG package of programs (Fromm *et al.*, 1990) and DNA Inspector II (Textco, W. Lebanon, NH, USA).

## Results

All of the constructs described here were generated by fusing the same 1992 bp *Pst*I fragment from the maize *Ubi-1* gene upstream of the relevant polylinker or marker sequence (Fig. 1). This *Ubi-1* *Pst*I fragment contains 899 bp of promoter sequence, 83 bp of 5' untranslated exon, and 1010 bp of first intron sequence, terminating through reconstitution of the *Pst*I site precisely at the G in the AG dinucleotide of the 3' splice junction of the intron (Christensen *et al.*, 1992). The nucleotide sequences at the fusion junctions at the 3' end of the *Ubi-1* DNA are shown for each construct in Fig. 2.

### pAHC17

This plasmid is a *Ubi-1* promoter expression vector for *Bam*HI (or *Bam*HI-compatible) cloning of protein coding regions. It contains the *Ubi-1* promoter, 5' untranslated region and intron upstream of an unique *Bam*HI site (Fig. 1). About 250-bp of nopaline synthase (NOS) 3' untranslated sequence and polyadenylation signals are located downstream of the *Bam*HI site. The 1992 bp *Pst*I fragment of the *Ubi-1* gene had been previously cloned into the *Pst*I site of M13mp19 for sequencing (Christensen *et al.*, 1992). A *Hind*III-*Bam*HI fragment from the replicative form of that clone was isolated and ligated to a 3175 bp *Hind*III-*Bam*HI fragment of pMF6 (Goff *et al.*, 1991) containing pUC8 sequence and 250 bp

of NOS 3' polyadenylation sequence adjacent to the *Eco*RI site.

The polylinker sequence is located between the end of the *Ubi-1* intron and the *Bam*HI cloning site and between the *Bam*HI site and the NOS sequence (Fig. 2). Thus, a reporter gene cloned into the *Bam*HI site is flanked by polylinker sequence on both the 5' and 3' sides. *Sal*I and *Xba*I sites from the M13mp19 polylinker are upstream of the *Bam*HI site and a *Sal*I and a *Pst*I site from the pUC8 polylinker are on the 3' side.

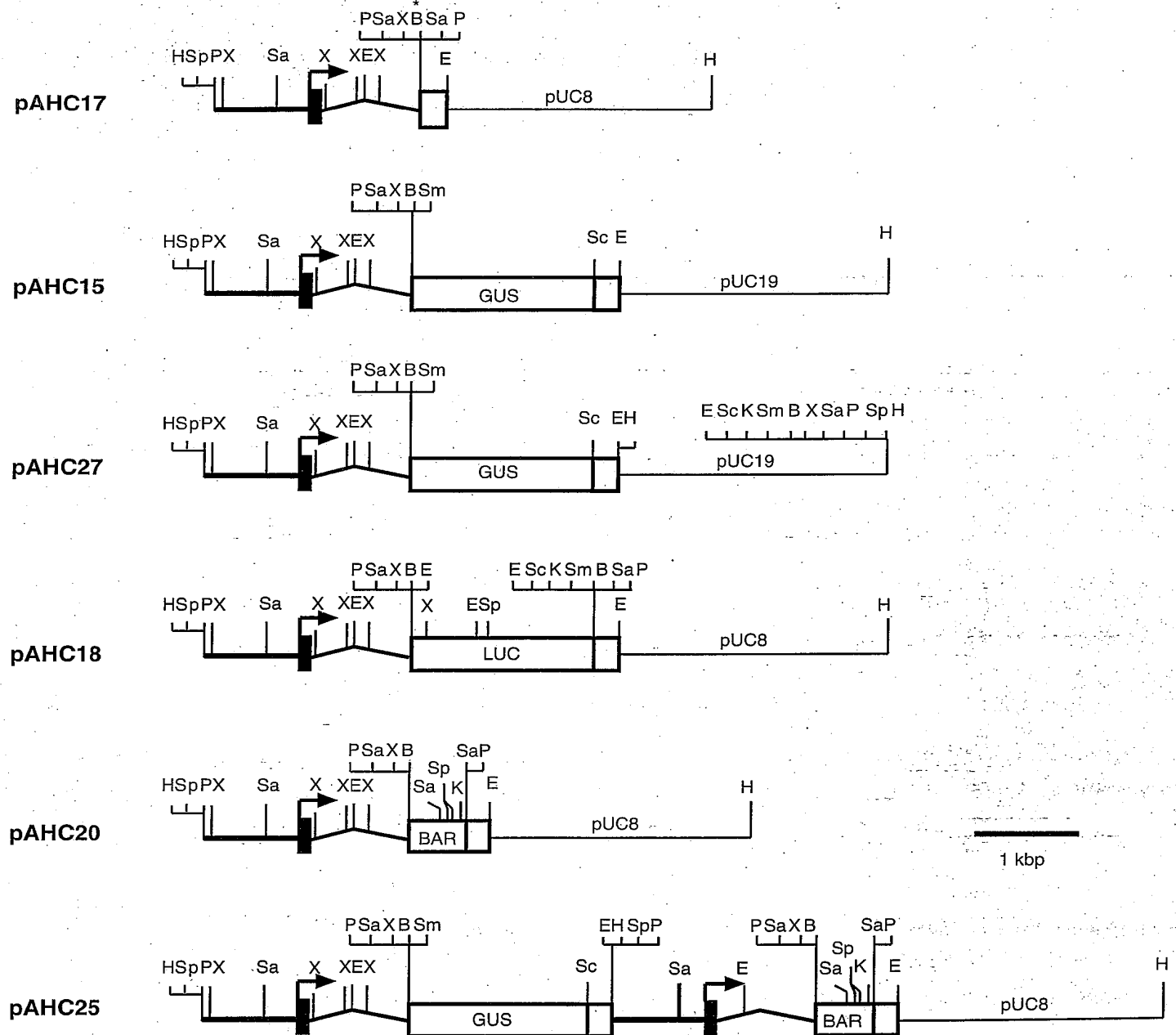
### pAHC15 and pAHC27 (pUbi-GUS)

These plasmids contain the maize *Ubi-1* promoter, 5' untranslated region and first intron fused to the coding region of the *E. coli uidA* gene (GUS) (Fig. 1). To produce pAHC15, *Hind*III-*Eco*RI fragment of pBI101.2 (Jefferson *et al.*, 1987) containing the *Hind*III to *Sma*I region of the pUC19 polylinker, the GUS coding sequence, and 260 bp of the nopaline synthase gene polyadenylation signal was cloned into the *Hind*III and *Eco*RI sites of pUC19 (pUC19-GUS-NOS). The 1992 bp *Pst*I fragment of the maize *Ubi-1* gene (Christensen *et al.*, 1992) was cloned into the *Pst*I site of the polylinker sequence upstream of the GUS coding sequence in pUC19-GUS-NOS. The construct contains the *Ubi-1* sequence in an orientation such that transcription will proceed through the ubiquitin 5' exon, intron and the GUS coding sequence, terminating in the NOS 3' sequence.

pAHC27 contains the same Ubi-GUS-NOS construct as pAHC15 but as a *Hind*III fragment cloned into the *Hind*III site of pUC19 (Fig. 1). This construct was generated to facilitate the production of pAHC25 (see below). The *Eco*RI site at the 3' end of the chimeric gene in pAHC15 is not unique as there is an additional *Eco*RI site in the *Ubi-1* intron. However, the *Hind*III site at the 5' end of the chimaeric gene is unique. To allow the entire construct to be removed as one fragment for further subcloning, a *Hind*III site was introduced at the 3' end of the chimaeric gene. This was achieved by partially digesting pAHC15 with *Eco*RI, optimizing the digestion for linear fragments. The *Eco*RI sites were filled in with dNTPs and Klenow fragment of DNA Polymerase and a *Hind*III linker (5' CAAGCTTG 3'; New England Biolabs) was added. Addition of the linker also restored the *Eco*RI site. The DNA was digested with *Hind*III to remove excess linker and to cut at the 5' end of the chimaeric gene. The 4.15 kb *Hind*III fragment containing the *Ubi-1 gus* chimaeric gene was gel-purified and subcloned into *Hind*III-digested pUC19. The chimaeric gene in the resultant pAHC27 is oriented such that the entire pUC19 polylinker is upstream of the *Ubi-1* promoter (Fig. 1).

### pAHC18 - pUbi-LUC

This plasmid contains the *Ubi-1* promoter-5' exon-first



**Fig. 1.** Schematic diagrams of expression vectors based on maize *Ubi-1* sequences. The relative sizes of the various segments of the linearized plasmids are drawn to scale. Bold straight line, *Ubi-1* promoter sequences; filled box, *Ubi-1* exon; angled line, *Ubi-1* intron; labelled open boxes, reporter gene sequences; blank open box, nopaline synthase 3' untranslated sequence; thin straight line, pUC8 (pAHC17, 18, 20, and 25) or pUC19 (pAHC15 and 27) sequence. Arrow at the *Ubi-1* exon signifies transcription start site and direction. GUS,  $\beta$ -glucuronidase (Jefferson *et al.*, 1987); LUC, firefly luciferase (Ow *et al.*, 1986); BAR, phosphinothricin acetyltransferase (De Block *et al.*, 1987). Restriction sites used in construction of the chimaeric genes and in adjacent polylinker sequences are shown. The *Bam*HI site marked with an asterisk in pAHC17 is a unique site for cloning *Bam*HI or *Bam*HI-compatible fragments. (Note: The *Xba*I sites located in the *Ubi-1* intron are subject to methylation interference in *dam*<sup>+</sup> *E. coli* strains. Also, although the *Eco*RI site in the *Ubi-1* intron upstream of the *gus* sequence in pAHC15 is cleaved efficiently, in both pAHC27 and pAHC25 the corresponding *Eco*RI site is cut very inefficiently). B, *Bam*HI; E, *Eco*RI; H, *Hind*III; K, *Kpn*I; P, *Pst*I; Sa, *Sal*I; Sc, *Sac*I; Sm, *Sma*I; Sp, *Sph*I; X, *Xba*I.

**pAHC17****UBI1 Intron****NOS 3'****Pst I Sal I Xba I Bam HI Sal I Pst I**

.....ctgcagGTCGACTCTAGAGGATCCGTCGACCTGCAG.....  
 .....gacgtcCAGCTGAGATCTCCTAGGCAGCTGGACGTC.....

**pAHC15 / 25 / 27****UBI1 Intron****GUS (from pBI101.2)****Pst I Sal I Xba I Bam HI Sma I**

.....ctgcagGTCGACTCTAGAGGATCCCGGGTAGTCAGTCCCTTATG.....  
 .....gacgtcCAGCTGAGATCTCCTAGGGGCCATCAGTCAGGGAATAC.....

**pAHC18****UBI1 Intron****LUC (from pDO432)****Pst I Sal I Xba I Bam HI**

.....ctgcagGTCGACTCTAGAGGATCCGAGCTTGGAAATTCCTTTGTGTACATTCTTGAATGTCGCTCGCAGTGACATTAGCATTCCGGTACTGTTGGTAAAATG.....  
 .....gacgtcCAGCTGAGATCTCCTAGGCTCGAACCTTAAGGAAACACAATGTAAGAATTACAGCGAGCGTCACTGTAATCGTAAGGCCATGACAACCATTTTAC.....

**pAHC20 / 25****UBI1 Intron****BAR (from pUC/BASTA)****Pst I Sal I Xba I Bam HI**

.....ctgcagGTCGACTCTAGAGGATCCATCGATTAGGAAGTAACCATG.....  
 .....gacgtcCAGCTGAGATCTCCTAGGTAGCTAATCCTTCATTGGTAC.....

**Fig. 2.** Nucleotide sequence of the polylinker region comprising the junction between the *Ubi-1* intron and the reporter gene or NOS 3' sequence. The *Ubi-1* intron sequence (lower case) ends with a *Pst*I site containing the 3' splice junction. The reporter gene sequences shown downstream of the polylinker are those upstream of the respective coding sequence and end with the ATG translation start codon (italicized) shown for each.

intron fused to a luciferase (LUC) reporter coding sequence (Fig. 1). An 1892 bp *Bam*HI fragment of pDO432 (Ow *et al.*, 1986) containing 80 nucleotides of 5' untranslated sequence, the luciferase coding region (1649 nucleotides) and 163 bp of 3' untranslated sequence was cloned into the unique *Bam*HI site of pAHC17. This construct contains the luciferase coding sequence in the same orientation as the ubiquitin promoter.

**pAHC20 - pUbi-BAR**

The Ubi-BAR chimaeric gene in this plasmid provides selection of transformants resistant to Basta<sup>TM</sup> herbicide (phosphinothricin) (De Block *et al.*, 1987). The Ubi-BAR construct was formed by ligating a 570 bp *Bam*HI-*Bcl*I fragment containing the *bar* gene into the *Bam*HI site of pAHC17. The *bar* gene fragment was excised from a plasmid (pUC8/BASTA) obtained from Dr M. Fromm (Fromm *et al.*, 1990). The resultant pAHC20 plasmid has *bar* in the same orientation as the maize *Ubi-1* promoter (Fig. 1). The construct contains 18 bp of sequence between the *Bam*HI site and the translation start codon

of the *bar* gene (Fig. 2). The *Bcl*I site is 11 bp downstream of the TGA stop codon.

The unique *Hin*dIII site at the 5' end of the *Ubi-1* sequence makes this plasmid very adaptable. This restriction site is suitable for insertion of a second chimaeric gene, such as a scorable marker also driven by a second *Ubi-1* promoter, as detailed below for pAHC25, or for any other desired promoter-gene combination.

**pAHC25 - pUbi-GUS/Ubi-BAR**

pAHC25 contains both a selectable marker (*bar*) and a scorable marker (GUS), each under the transcriptional control of a separate *Ubi-1* promoter (Fig. 1). The two chimaeric genes were first assembled separately in pAHC20 and pAHC27 and then the double construct was formed. This was achieved by excising the Ubi-GUS-NOS-containing *Hin*dIII fragment from pAHC27 and subcloning it into *Hin*dIII-digested pAHC20. The resultant pAHC25 plasmid has both Ubi-BAR and Ubi-GUS chimaeric genes in the same orientation.



## Discussion

The high activity of the maize *Ubi-1* promoter has now been documented in transient and/or stable transformation configurations in a number of monocot systems including rice (Bruce *et al.*, 1989; Toki *et al.*, 1992; Cornejo *et al.*, 1993; Uchimiya *et al.*, 1993; Takimoto *et al.*, 1994), wheat (Taylor *et al.*, 1993; Weeks *et al.*, 1993), barley (Wan and Lemaux, 1994), sugarcane (Gallo-Meagher *et al.*, 1993; Taylor *et al.*, 1993), maize (Christensen *et al.*, 1992; Gallo-Meagher *et al.*, 1993) *Pennisetum* (Taylor *et al.*, 1993), *Panicum* (Taylor *et al.*, 1993) and *Lemna* (Rolfe and Tobin, 1991). Whether or not the high level of expression of selectable marker genes fused to *Ubi-1* actually increases the efficiency of recovery of fertile transgenic plants relative to less active promoters like that from the CaMV 35S gene is yet to be rigorously examined (see Wan and Lemaux, 1994). However, the high level of GUS expression provided by the *Ubi-GUS* constructs has proven valuable in enabling rapid histochemical screening of transformants for transgene activity (Cornejo *et al.*, 1993).

The original intron present in the 5'-untranslated region of the *Ubi-1* gene (Christensen *et al.*, 1992) was retained in all the constructs here because of numerous previous studies showing that introns frequently strongly enhance transgene expression in cereals (Callis *et al.*, 1988; Bruce and Quail, 1990; McElroy *et al.*, 1990; Vasil *et al.*, 1993). The influence of the *Ubi-1*-intron has not been tested directly, but there is evidence that this maize sequence is spliced correctly in transgenic rice cells (Toki *et al.*, 1992).

Detailed examination of the spatial and temporal expression patterns of the *Ubi-1* promoter in transgenic plants is yet to be reported. However, initial data with a *Ubi-gus* construct indicate expression in all organs of transgenic rice consistent with a potential for targeting a wide spectrum of cells (Cornejo *et al.*, 1993; Takimoto *et al.*, 1994).

An additional potentially useful feature of the *Ubi-1* promoter is that it is stress-inducible. Both thermal and mechanical stress have been shown to cause a strong enhancement of the *Ubi-gus* transgene activity in transformed rice (Cornejo *et al.*, 1993; Takimoto *et al.*, 1994). It is possible that this fact may result in stronger expression of selectable marker fusion genes during the early stages of transformation, where recipient cells are exposed to a variety of stresses such as high osmotic pressures, particle bombardment and growth on toxic compounds. A subsequent decrease in expression level is expected upon removal of the selective conditions so that regenerated transgenic plants would presumably not continue to express the marker at high levels when it is no longer needed. The stress-inducibility of the *Ubi-1* promoter might also be useful for driving conditional

expression of genes that confer tolerance or resistance to various biotic and abiotic stresses such as pathogen attack, heat and water deficit (Takimoto *et al.*, 1994).

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Initiate calli from transgenic ~~plant~~ seeds (from To plants of 4R-VIO-28 and 4R-VIO-67).

De-husk, sterilize with ethanol for ~ 1 min, then 25% bleach for ~ 25 min. Wash 4 times with H<sub>2</sub>O. place on NB. keep at 27°C in darkness.

Stain the tips of shoots for GUS:

VIO-28: 150 total, 32 GUS-negative

VIO-67: 108 total, 25 GUS-negative.

cut the scutellum, and place on fresh NB medium. keep at 27°C.

transformation:

same procedure as before. Use 15 ml NB-AS to wash off 2-day old bacterium from each plate (LB-RS). The calli were from 2 to 3 subcultures. GUS expression is good for both VIO-28 and VIO-67. Separate two lines for the experiments.

Constructs:

- |               |   |   |                                   |
|---------------|---|---|-----------------------------------|
| AGL2: pMBU223 | — | ① | Ubi'-tm1'                         |
| " : " 225     | — | ② | Ubi'-JAMV-GUSd-tm1' (NO STOP)     |
| " : " 227     | — | ③ | Ubi'-JAMV-JAMV-GUSd-tm1'          |
| " : " 229     | — | ④ | Ubi'-JAMV-JAMV-iCAT-GUSd-tm1'     |
| " : " 231     | — | ⑤ | Ubi'-JAMV-JAMV-iCAT-GUSd(AS)-tm1' |
| " : " 237     | — | ⑥ | Ubi'-JAMV-GUSd-GUSS(AS)-tm1'      |
| " : " 239     | — | ⑦ | Ubi'-JAMV-GUSd-GUSS'(AS)-tm1'     |

Wash the calli with H<sub>2</sub>O-Timentin twice, blot on filter paper, then transfer to NB-Timentin 150-Hygromycin 50-Halaphos 5. Keep at 27°C.

Signature of Researcher

Miguel Wong

Date

Signature of Supervisor

W. T. Yang

Date

Experiment:

Date: / /

Transfer all calli to NB-Timentin 150 - hygromycin 50  
- bialaphos 10 mg/L.

Transfer all calli to NB-Timentin 150 - hygromycin 25 -  
bialaphos 10 mg/L.

1) Transfer resistant callus pieces to NB-Timentin 150 - hygromycin  
25 - bialaphos 10 mg/L.

Constructs / Lines	Total number of callus pieces	No of bialaphos resistant callus.
1a: pMBW223/V10-28	115	25
1b: pMBW223/V10-67	71	36
2a: pMBW225/V10-28	124	26
2b: pMBW225/V10-67	63	25
3a: pMBW227/V10-28	122	20
3b: pMBW227/V10-67	77	22
4a: pMBW229/V10-28	<del>86</del> 113	30
4b: pMBW229/V10-67	73	24
5a: pMBW231/V10-28	161	22
5b: pMBW231/V10-67	88	36
6a: pMBW233/V10-28	125	21
6b: pMBW233/V10-67	79	21
7a: pMBW239/V10-28	193	28
7b: pMBW239/V10-67	116	23

For these lines which ~~had~~ proliferated well  
and produced relatively more number of callus pieces,  
transfer part of the callus pieces to NB-TURO B5, and keep  
part of them on the old plates. Also stain some <sup>of V10-67 derived</sup> for cells  
look promising.

1b: lines 1, 2, 3, 5, 7, 8, 10, 11, 12, 13, 15, 17, 19, 23, 25. All are <sup>Ⓢ</sup> strong.

2b: lines 4, 5, 10, 11, 14. 10 not blue; 4, 14 weak; 5, 11 strong.

3b: <sup>W+S</sup> 1, 3, 5, 6, 7, 8, 9, 10, 16, 18. S - strong; ⊖ - negative; W - weak;  
W+S - A piece is strong, the rest is weak.

Signature of Researcher: Highs Wang 2

Date: / /

Signature of Supervisor: Mc Tay

Date: / /

Experiment:.....

Date: / /

4b: lines:

(3) (4) (5) (7) (8) (9) (12) (14) (16) (17) A blue spot

5b:

lines (3) (4) (8) (9) (14) (17) (19) (23) (24) (?) A blue spot

6b: lines

(2) (4) (6) (8) (9) (10) (11) (13) (14) (15) very weak stw

7b: lines

(2) (3) (5) (6) (10) (13) (14) (15) A relatively big blue spot. callus missing

Transfer some good callus pieces onto PRT15H25BS.

Transfer remaining good callus pieces into PRT15H25BS or PRT15H25BS.

Note: The medium missed glutamine.

Transfer call the calli transferred to PRT15H25BS on to RTH20BS. Keep under light at 24°C.

Stain the callus pieces for GUS. — see result next attached page.

Some pieces also stained on

Scan file

plate.tif

GUS-negative or weak lines:

2a: 3, 7, 10, 12  
blue spotBrightness: 180  
Contrast: 178  
Scaling 400%

3a?: 2, 4 (one blue pieces one white pieces)

4a?: 5, 7

5a?: none.

6b: 4, 6, 8, 9, 12

6a?: 1, 3

7b: 5, 3, 6

7a: 4, 8, 9

2b: 1, 10, 12

3b: 1, 2, 3, 12, 18

4b: 7, 8, 11, 12, 16, 17

5b: none

Signature of Researcher

Date: / /

Signature of Supervisor

Date: / /

scan file: plat01.tif

	S	S	S	4	5	7	5	16	1	2	3	S
10a.	1	2	3	8	9	10	11	12	13	14	15	16
1b	5	6	7	21	24	25						
1c	18	19	20	23	26	27	28	29	30	31	32	33
2a	1	2	3	4	5	6	7	8	9	10	11	12
2	14	15	16	17	18	19	20	21	22	23	24	25
3a?	1	2	3	4	5	6	7	8	9	10	11	12
4a?	1	2	3	4	5	6	7	8	9	10	11	12

S = strong  
M = medium  
Θ = negative  
W = weak.

Scan file: platb2.tif

plate (II)

4a	4b	1	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100
4a	4b	1	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100

Scan file: platb3.tif

plate (III)		Scan file:		platb3.tif.																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																											
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179

Experiment

Date / /

Trans for all good calli from PRTH-B5 to RTH-B5.

Stain the following for GUS:

- 1a → 1, 2, 3, 4, 5, 7, A 1b → 1, 5, 7, 10, 11  
12, 16, 17, 19, 23, 25 — All blue
- 2a → 1, 4\*, 5, 8, 10, 12, 13, 14, 15, 16, 18, 4b →  
10, 4, 5, 12, 14, 15, 16, 18, 19, 23, 25 — very weak or GUS ⊖
- 3a? → 1, 2, 3, 4, 6, 7, 8, 3b → 1, 2, 3, 16, 18
- 4a? → 1, 2, 3, 4, 5, 6, 7, 9, 10, 11, 4b → 8, 10  
11, 12, 13, 14, 15, 17
- 5a? → 1, 4, 6, 8, 9, 10, 12, 15, 16, 17, A
- 6a → 1, 3, 4, A, 6b → 4, 5, 9, 10, 11, 13, 14  
15, 16
- 7a → 2, 4, 6, 7, 8, 9, 10, 11, A, 7b → 2, 5, 6  
10, 11, 13, 14, 15

⊖ — very weak GUS

⊖ — basically GUS ⊖

Stain ~~all~~ callus pieces from all lines on regeneration media. These are all the callus lines obtained.

Keep the rest for regeneration.

GUS assay — see page 55, book 5.

Signature of Researcher

Date / /

Signature of Supervisor

Date / /



Project

(code)

Book No:

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Experiment Title

GUS Assay — super-transformed rice calli

Materials: choose healthy callus pieces from each line (normally <sup>a bit</sup> green) put into <sup>2x</sup>Eppendorf. Keep one Eppendorf at RT for protein extraction keep the other in  $-70^{\circ}\text{C}$  freezer for DNA & RNA isolations. Also place a few smaller callus piece from ~~most~~ most of the lines in NB medium for proliferation. All the calli were on regeneration medium (for a few weeks). See page 178 of book 4 for more details about the callus materials.

Protein Extraction = Follow the method on page 61 of the Thesis. Weigh the calli, add equal amount of the extraction buffer to each Eppendorf (i.e. 100  $\mu\text{g}$  to 100  $\mu\text{m}$ ). Grind with the machine for  $\sim 10$  sec at  $\sim 200$  rpm. Keep on ice first and then at  $-70^{\circ}\text{C}$ . The next day thaw the slurry and spin twice and collect the supernatant. ~~Keep~~ Store at  $-70^{\circ}\text{C}$ .

Protein Quantification:

Add 100  $\mu\text{L}$  H<sub>2</sub>O to a cuvette, then add 1  $\mu\text{L}$  of the sample, followed by 200  $\mu\text{L}$  BioRAD ~~reagent~~ reagent solution. Mix and measure the OD at 595 nm. make a standard curve using BSA.

Results: see the attached pages

GUS Assay = Follow the method in the thesis: add

9  $\mu\text{L}$  extraction buffer, 3  $\mu\text{L}$  sample and 10  $\mu\text{L}$  reaction buffer to each well on the microtiter plate. Incubate at  $37^{\circ}\text{C}$  for  $\sim 40$  min. Stop the reaction by adding 180  $\mu\text{L}$  0.2 M Na<sub>2</sub>CO<sub>3</sub> to each well. Measure with FluoroScan. The reaction solution is 4.2 mg MUG in 10 ml extraction buffer.

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Print Name ..... Date .....

Supervisor

Date .....

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Project

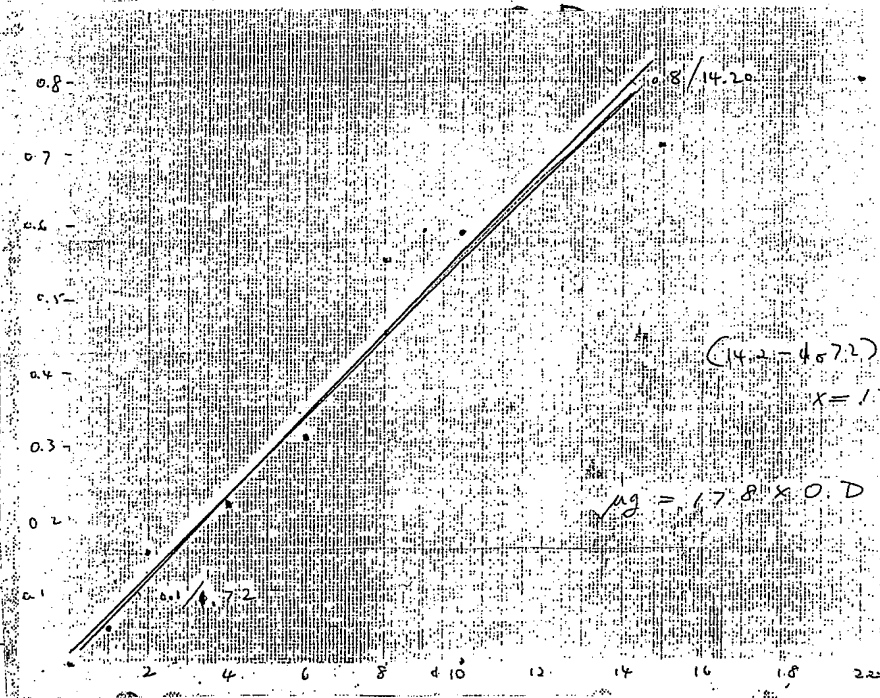
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Book No:

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Experiment Title

BSA	0.0.
1 ml -	.09
2 ml -	.15
4 ml -	.22
6 ml -	.31
8 ml -	.45
10 ml -	.59
15 ml -	.71
20 ml -	.80
25 ml -	1.02



		0.0	concentration (mg/ml)		
1a1 -	.49	8.7	10/612 -	.56	10.0
1a2 -	.53	9.4	11/613 -	.54	9.6
1a3 -	.55	9.8	12/614 -	.48	8.5
1a4 -	.53	9.4	1/615 -	.44	7.8
1a5 -	.52	9.2	2/616 -	.40	7.1
1a6 -	.42	7.5	3/617 -	.47	8.4
1a7 -	.53	9.4	4/618 -	.49	8.7
1aA -	.59	10.5	5/619 -	.49	8.7
1aB -	.56	10.0	6/620 -	.45	8.0
1aC -	.61	10.8	7/620* -	.51	9.1
1b1 -	.29	5.2	8/623 -	.37	6.6
1b2 -	.41	7.3	9/624 -	.43	7.6
1/63 -	.21	3.7	10/625 -	.46	8.2
2/64 -	.36	6.4	11/2a1 -	.24	4.3
3/65 -	.31	5.5	12/2a2 -	.30	5.3
4/66 -	.49	8.7	1/2a3 -	.41	7.3
5/67 -	.27	4.8	22/24 -	.56	10.0
6/68 -	.46	8.2			

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Recorded by	Print Name	Supervisor	Date	Signed
7/69 -	8/610 -	8/611 -	32	5.3

## Experiment

3205- .53 9.4	11302- .25 4.8
4206- .44 7.8	12303- .18 3.2
5207- .55 9.8	1304- .18 3.2
6208- .65 11.6	2305- .21 3.7
7209- .41 7.3	3306- .25 4.4
82010- .51 9.1	4307- .26 4.6
42011- .50 8.9	5308- .19 3.4
02012- .32 5.7	6309- .26 4.6
12013- .30 5.3	730A- .28 6.8
13014- .36 6.4	8301- .34 6.0
2015- .33 5.9	9302- .55 9.8
22016- .44 7.8	10303- .31 5.5
32017- .29 5.2	11305- .32 5.7
42018- .32 5.7	12306- .35 6.2
520A- .62 11.0	1307- .42 7.5
6201- .33 5.9	2308- .29 5.2
7202- .32 5.7	3309- .23 4.1
8203- .47 8.4	43010- .26 4.6
9204- .54 9.6	53011- .31 5.5
10205- .52 9.2	13012- .32 4.3
11206- .57 10.1	73014- .33 5.9
12207- .44 7.8	83016- .62 11.0
13209- .24 4.3	93017- .55 9.8
122010- .27 4.8	103018- .29 5.2
22011- .39 6.9	11401- .32 5.7
42012- .21 3.7	12402- .22 3.9
52014- .41 7.3	1403- .42 7.5
62016- .27 4.8	2404- .31 5.5
72017- .31 5.5	3405- .31 5.5
82019- .31 5.5	4406- .32 5.7
920A- .24 4.3	5407- .29 5.2
10301- .18 3.2	6408- .36 6.4

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DateRead and Understood by  
Date

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Project

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Book No:

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Experiment Title

First Reading of the Gas Assay.

	1	2	3	4	5	6	7	8	9	10	11	12
A	14934	14754	14783	14722	14669	14980	14311	14951	14855	14794	15286	14587
B	15325	14732	14841	14719	15070	14584	14476	14564	14648	14752	14772	14639
C	15189	14616	14617	14618	14619	14620	14621	14622	14623	14624	14625	14626
D	2013	2014	2015	2016	2017	2018	2019	2020	2021	2022	2023	2024
E	2015	2016	2017	2018	2019	2020	2021	2022	2023	2024	2025	2026
F	2015	2016	2017	2018	2019	2020	2021	2022	2023	2024	2025	2026
G	2015	2016	2017	2018	2019	2020	2021	2022	2023	2024	2025	2026
H	2015	2016	2017	2018	2019	2020	2021	2022	2023	2024	2025	2026

ate No 1

	1	2	3	4	5	6	7	8	9	10	11	12
A	4002	4004	4005	4006	4007	4008	4009	4010	4011	4012	4013	4014
B	4002	4004	4005	4006	4007	4008	4009	4010	4011	4012	4013	4014
C	4002	4004	4005	4006	4007	4008	4009	4010	4011	4012	4013	4014
D	4002	4004	4005	4006	4007	4008	4009	4010	4011	4012	4013	4014
E	4002	4004	4005	4006	4007	4008	4009	4010	4011	4012	4013	4014
F	4002	4004	4005	4006	4007	4008	4009	4010	4011	4012	4013	4014
G	4002	4004	4005	4006	4007	4008	4009	4010	4011	4012	4013	4014
H	4002	4004	4005	4006	4007	4008	4009	4010	4011	4012	4013	4014

ate No 2

TRANSF. 4:

	1	2	3	4	5	6	7	8	9	10	11	12
A	273.2	283.9	283.9	240.4	23.20	0.094	0.094	0.204	0.408	0.173	0.141	0.377
B	88.46	116.3	116.3	218.1	0.173	0.188	0.566	0.157	0.157	0.188	0.110	
C	147.5	97.77	97.77	46.28	134.9	0.188	0.204	0.283	0.833	0.314	0.518	0.126
D	46.48	61.26	61.26	121.1	78.18	0.220	0.267	0.220	0.314	0.236	0.440	0.534
E	384.9	484.0	484.0	207.5	14.00	0.157	0.314	0.550	0.283	0.393	0.141	0.141
F	410.4	503.1	503.1	128.8	12.35	0.126	0.094	0.283	0.408	0.660	3.236	0.188
G	999.4	36.74	36.74	31.40	9.033	0.157	0.314	0.503	0.440	0.110	0.094	0.126
H	525.7	32.17	32.17	153.0	9.001	0.173	0.188	0.236	0.110	0.408	0.157	0.220

ate No 3

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Print Name ..... Date .....Supervisor  
Date .....Read and Understood by  
Print Name ..... Date .....

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## Project

Experiment

4a9- .27 7 4.8	3 5a5- .37 6.6
4a10- .36 8 6.4	4 5a6- .32 5.7
4a11- .42 9 7.5	5 5a7- .29 5.2
4a12- .25 10 4.45	6 5a8- .28 5.0
4aA- .53 11 9.43	7 5a9- .36 6.4
4aB- .59 12 10.5	8 5a10- .18 3.2
4aC- .56 10.0	9 5a11+12- .21 3.7
4aD- .50 2 8.9	10 5a12- .22 3.9
4aX- .18 3 3.2	11 5a13- .25 4.4
4aY- .18 4 3.2	12 5a14- .21 3.7
4aZ- .22 5 3.9	1 5a15- .17 3.0
4b1- .36 6 6.4	2 5a16- .27 4.8
4b3- .47 7 8.4	3 5a17- .32 5.7
4b4- .30 8 5.3	4 5aA- .25 4.4
4b5- .49 9 8.7	5 5b1- .58 10.3
4b6- .22 10 3.9	6 5b2- .35 6.2
4b7- .20 11 7.1	7 5b3- .37 6.6
4b8- .36 12 8.8	8 5b4- .43 7.6
4b9- .36 1 6.4	9 5b5- .32 5.7
4b10- .38 2 6.7	10 5b6- .32 5.7
4b11- .29 3 5.2	11 5b8- .52 9.2
4b12- .26 4 4.6	12 5b9- .37 6.6
4b13- .25 5 4.4	1 5b10- .17 3.0
4b14- .27 6 4.8	2 5b12- .20 3.6
4b15- .46 7 8.2	3 5b13- .13 2.3
4b16- .42 8 7.5	4 5b14- .30 5.3
4b17- .48 9 8.5	5 5b15- .39 6.4
4b18- .39 10 6.9	6 5b16- .21 3.7
5a1- .40 11 7.1	7 5b17- .20 3.6
5a2- .41 12 7.3	8 5b19- .44 7.8
5a3- .36 1 6.4	9 5b20- .30 5.3
5a4- .38 2 6.8	10 5b22- .16 2.8

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Experiment Title

5b23- .24 11 4.3	7 7a7- .20 3.6
5b24- .31 12 5.5	8 7a8- .26 4.6
5b26- .18 13 2.	9 7a9+10- .23 4.1
5b27- .25 24 4.	10 7a10- .20 3.6
5b29- .22 33 9	11 7a11- .27 4.8
5b30- .16 42 8	12 7a12- .23 4.1
6a1- .32 55 7	1 7a14- .35 6.2
6a3- .27 64 8	2 7aA- .24 4.3
6a4- .36 76 4	3 7aB- .23 4.1
6aA- .32 85 7	4 7aC- .16 2.8
6aB- .34 96 0	5 7b2- .35 6.2
6aC- .33 10 5.9	6 7b3+5- .39 6.9
6b1- .34 11 6.0	7 7b3- .21 3.7
6b2- .30 12 5.3	8 7b5- .35 6.2
6b3- .25 1 4.4	9 7b6- .34 6.0
6b4- .24 2 4.3	10 7b7- 7aA .26 4.6 7
6b5- .39 3 6.9	11 7b10- .22 3.9
6b6- .26 4 4.6	12 7b11- .39 6.9
6b8- .31 5 5.5	A 1 7b13- .34 6.0
6b9- .34 6 6.0	B 2 7b14- .34 6.0
6b10- .43 7 7.6	C 3 7b15- .25 4.4
6b11- .32 8 5.7	D 4 7b16- .30 5.3
6b13- .28 9 5.0	E 5 Controls N°1- .44 7.8
6b14- .52 10 9.2	F 6 Controls N°2- .55 9.8
6b15- .45 11 8.0	G 7 extraction
6b16- .31 12 5.5	H 8 extraction
7a1- .29 1 5.2	
7a2- .24 2 4.3	
7a3- .26 3 4.6	
7a4- .26 4 4.6	
7a5- .24 5 4.3	
7a6- .27 6 4.8	

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DateRead and Understood by  
Print Name

Date

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Initialed

Signed

## Experiment Title

plate 1  
second reading

	1	2	3	4	5	6	7	8	9	10	11	12
A	4790	4695	4711	4677	4598	4634	4143	4650	4539	4596	4454	4386
B	4672	4550	4523	4702	4530	4503	4405	4514	4613	4647	4730	4465
C	4996	4710	4876	4862	4768	4693	4725	4790	4566	4631	4695	3041
D	4856	161.5	223.3	3355	994.1	4653	4648	4642	3421	4651	4668	4444
E	4832	4712	103.6	4771	4071	4670	4593	4610	1610	4439	1566	152.1
F	708.2	4645	4641	4823	4577	4545	4638	523.4	326.8	4532	4404	4402
G	389.6	4530	4598	4364	4461	4271	4315	62.14	87.80	15.52	4395	5851
H	4639	3851	4559	4585	4551	280.1	123.1	4429	4459	4453	4361	4320

TRANSF. 4:

plate 2  
second reading

	1	2	3	4	5	6	7	8	9	10	11	12
A	4729	4525	314.7	4914	1075	4125	4789	4619	4645	4587	4693	4614
B	4614	4771	4775	4793	4766	4766	4709	4565	4711	212.7	4628	4497
C	4606	4743	314.5	4835	110.6	108.0	4651	355.5	4667	4607	390.5	1060
D	4725	4867	4752	4870	4861	4717	3929	152.7	4562	4698	4613	4616
E	4473	277.1	3912	4771	1696	4596	4572	4311	366.4	4429	4501	4466
F	325.9	4678	4684	4732	4658	4704	4650	1340	2678	4486	4590	4438
G	4539	3086	4705	256.5	959.3	494.8	4577	4596	673.9	72.50	4473	4497
H	621.2	4503	4578	162.2	406.1	197.1	4532	438.9	4499	4525	4304	216.6

plate 3  
second reading

	1	2	3	4	5	6	7	8	9	10	11	12
A	280.6	293.8	8.758	245.8	23.66	0.084						
B	90.59	119.1	8.758	4959	222.9	0.084						
C	149.7	99.73	8.758	47.30	137.9	0.174						
D	44.14	61.30	8.758	124.0	80.24	0.101						
E	382.3	4819	8.758	211.5	14.74	0.185						
F	409.2	4990	8.758	130.5	17.27	0.168						
G	961.6	36.46	8.758	31.88	9.377	0.084						
H	5027	31.89	8.758	154.4	9.024	0.134						

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Project

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Book No:

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Experiment Title

plate 1  
Second Reading  
2 x dilution

	1	2	3	4	5	6	7	8	9	10	11	12	
A	2836	2682	2873	2792	2805	2762	2469	2803	2718	2699	2637	2555	✓
B	2691	2595	2719	2872	2785	2691	2680	2713	2812	2733	2890	2628	✓
C	2973	2779	3001	2982	2980	2892	2944	2942	2776	2770	2845	1670	✓
D	2857	72.48	107.9	1845	486.0	2801	2888	2834	1930	2785	2885	2669	✓
E	2839	2838	48.92	2896	2357	2865	2791	2790	831.8	2661	808.5	87.91	✓
F	328.4	2713	2826	3024	2797	2807	2929	264.5	166.1	2817	2740	2680	✓
G	174.1	2624	2841	2620	2652	2579	2559	30.00	60.88	53.34	2730	4092	✓
H	2684	2110	2839	2758	2716	134.0	59.37	2696	2700	2705	2628	2594	✓

plate 2  
Second Reading  
2 x dilution

	1	2	3	4	5	6	7	8	9	10	11	12	
A	2797	2643	175.8	2976	543.5	2379	2906	2749	2796	2752	2820	2709	✓
B	2682	2837	2883	2916	2885	2900	2842	2690	2868	124.1	2785	2665	✓
C	2691	2833	178.5	2902	76.92	76.45	2800	189.8	2793	2764	207.0	522.1	✓
D	2752	2927	2935	2988	6607	2870	2282	94.69	2723	2861	2807	2777	✓
E	2552	152.4	2268	2978	837.9	2714	2710	2495	187.4	2635	2717	2594	✓
F	174.4	2896	2893	2870	2820	2888	2836	678.0	1426	2705	2809	2657	✓
G	2641	1677	2855	144.3	481.9	257.3	2757	2779	340.6	58.74	2695	2716	✓
H	310.4	2684	2750	101.6	213.7	116.2	2728	231.0	2689	2731	2557	124.3	✓

plate 3  
Second Reading  
2 x dilution

	1	2	3	4	5	6	7	8	9	10	11	12	
A	152.8	160.8	<del>31.1</del>	140.6	38.10								✓
B	68.14	82.42	<del>31.1</del>	2969	131.9								✓
C	94.68	73.50	<del>31.1</del>	49.31	92.04								✓
D	47.13	56.39	<del>31.1</del>	33.82	66.42								✓
E	197.5	2791	<del>31.1</del>	125.9	34.74								✓
F	211.9	2972	<del>31.1</del>	89.95	36.26								✓
G	459.2	44.20	<del>31.1</del>	43.16	32.10								✓
H	2859	41.82	<del>31.1</del>	100.4	31.91								✓

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## Experiment Title

Calibrated AUS Assay Reading.

Note: Figures from the second reading were used. For large figures use the readings from 2x dilution. For small ones, use the reading without dilution. The readings were then divided by the calculated concentration of each sample. Final data

Sample	calibrated reading	Sample	Calibrated Reading
1a1	650	1b15	762
1a2	571	1b16	783
1a3	586	1b17	714
1a4	594	1b18	686
1a5	680	1b19	685
1a6	736	1b20	723
1a7	525	1b20*	647
1aA	534	1b23	891
1aB	544	1b24	730
1aC	500	1b25	676
1b1	1014	2a1	1323
1b2	700	2a2	630

1b3	145-K	2a3	783
1b4	811	2a4	1420 16.2
1b5	929	2a5	23.8
1b6	660	2a6	473
1b7	1160	2a7	99.2
1b8	656	2a8	483
1b9	616	2a9	791
1b10	1023	2a10	623
1b11	987	2a11	433
1b12	547	2a12	977

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Experiment Title

Sample	Calibrated Reading	Sample	Calibrated Reading
2a15	962	3a4	121
2a16	728	3a5	1418
2a17	79.9	3a6	1291
2a18	1016	3a7	1139
2a19	428	3a8	1560
2b1	971	3a9	1121
2b2	979	3aA	752
2b3	664	3b1	10.4
2b4	168	3b2	8.96
2b5	578	3b3	2.82
2b6	155	3b5	958
2b7	19.5	3b6	1320
2b9	164	3b7	716
2b10	1130	3b8	811
2b11	819	3b9	1384
2b12	1634	3b10	1199
2b14	766	3b11	988
2b16	1169	3b12	65.1
2b17	1065	3b14	20.9
2b19	95.2	3b16	490
2bA	76.0	3b17	551
3a1	1760	3b18	1040
3a2	1245	4a1	922
3a3	1675	4a2	1330

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## Experiment Title

Sample	Calibrated Reading	Sample	Calibrated Reading
4a3	745	4b9	841
4a4	961	4b10	846
4a5	57.2	4b11	60.5
4a6	1044	4b12	1261
4a7	208	4b13	25.1
4a8	743	4b14	22.5
4a9	1210	4b15	683.
4a10	859	4b16	47.4
4a11	746	4b17	657
4a12	1223	4b18	801
4aA	598	5a1	55
4aB	516	5a2	145
4aC	536	5a3	860
4aD	637	5a4	861
4aX	1801	5a5	889
4aY	1822	5a6	1048
4aZ	1479	5a7	2541 ✓
4b1	906	5a8	1148
4b3	677	5a9	713
4b4	1015	5a10	47.7
4b5	659	5a11+12	1472
4b6	54.5	5a12	1467
4b7	784	5a13	1275
4b8	833	5a14	1501

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Experiment Title

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Sample	Calibrated Reading	Sample	Calibrated Reading
5a15	170.1	5b26	1650
5a16	57.7	5b27	762
5a17	795	5b29	1464
5aA	1353	5b30	91.6
5b1	165	6a1	168.3
5b2	875	6a3	103.1
5b3	821	6a4	861.
5b4	656	6aA	975
5b5	64.3	6aB	112
5b6	924	6aC	12.3 ✓
5b8	590	6b1	898
5b9	786	6b2	1024
5b10	109	6b3	141.
5b12	1609	6b4	1248
5b13	2515	6b5	797
5b14	1083	6b6	35.3 ✓
5b15	817	6b8	77.8
5b16	1561	6b9	32.8 ✓
5b17	1575	6b10	718
5b19	172	6b11	77.0
5b20	538	6b13	1076
5b22	1932	6b14	594
5b23	1306	6b15	639
5b24	966	6b16	39.3

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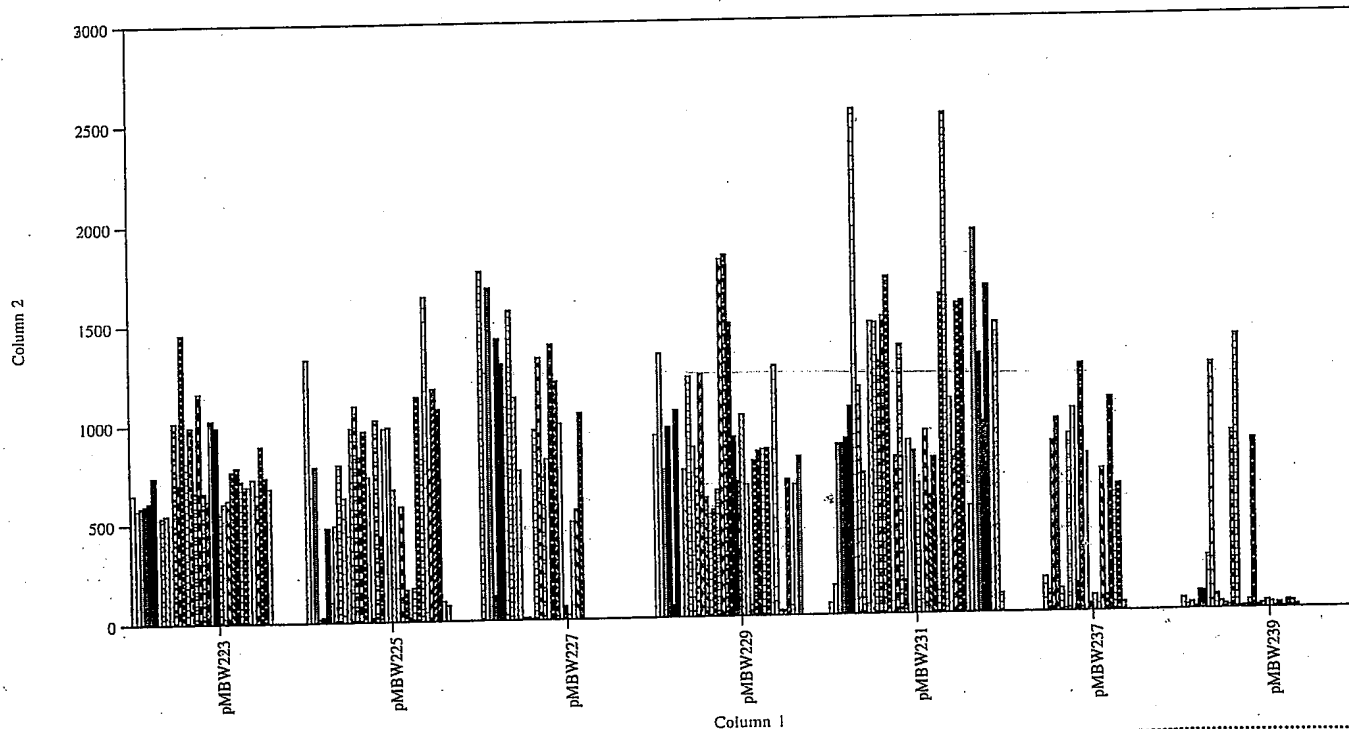
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## Experiment Title

Sample	Calibrated Reading	Sample	Calibrated Reading
7a1	54.0	7b2	39.6
7a2	21.1	7b3+5	160
7a3	32.5	7b3	12.8
7a4	9.60	7b5	20.0
7a5	88.9	7b6	35-2
7a6	85.2	7b7	28-4
7a7	267.1	7b10	8-17
7a8	1243	7b11	22-4
7a9+10	76.6	7b13	3-94
7a10	33.1	7b14	37-2
7a11	20.8	7b15	31-3
7a12	15.0	7b16	15-1
7a14	900.8	control 1	1.89
7aA	1382	control 2	1.76
7aB	8.90	exh. buff.	
7aC	11.4	exh. buff.	

## GUS ASSAY



Project

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(code)

Book No:

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Experiment Title Repeat the AUS Assay for supertransformed Vire cell  
this time dilute the extract to 1 mg/ml protein, use 1.5 ml for  
assay. Measure kinetic value rather than end product

NSF. 2:

NSF. 3:

NSF. 4:

plate No. 1

	1	2	3	4	5	6	7	8	9	10	11	12
A	121.0	45.58	99.28	26.17	92.21	108.8	6.432	90.80	98.24	93.76	109.5	109.5
B	30.35	40.04	165.0	23.05	167.2	118.5	11.27	110.6	19.29	121.0	115.1	77.10
C	106.1	73.12	29.97	50.11	80.34	117.8	10.26	193.523	16.24	154.5	21.1	2.22
D	71.60	0.224	0.321	5.290	0.946	32.44	128.8	31.28	5.023	40.27	36.40	53.24
E	29.15	89.06	0.256	68.23	5.841	93.43	73.19	128.1	1.506	203.8	1.275	2.67
F	0.209	42.05	117.5	118.9	21.44	155.0	190.9	0.773	0.146	38.43	64.16	149.2
G	0.955	68.32	105.6	136.6	140.4	62.38	17.79	0.199	0.197	34.57	128.0	130.3
H	196.2	12.43	140.0	90.13	157.4	0.518	0.231	116.1	43.18	31.66	38.88	115.5

NSF. 2:

NSF. 3:

NSF. 4:

plate No. 2

	1	2	3	4	5	6	7	8	9	10	11	12
A	133.0	98.46	0.502	39.35	1.545	10.36	111.6	14.42	88.06	52.28	30.26	107.5
B	26.75	105.1	135.1	95.04	71.15	31.82	58.08	56.32	44.62	0.454	17.32	73.20
C	20.32	107.4	0.453	87.81	0.299	0.206	12.47	0.21	11.15	19.22	0.299	0.52
D	37.20	53.94	105.5	56.73	60.36	71.12	13.17	0.424	26.98	115.5	149.7	66.75
E	145.8	0.534	9.400	35.33	1.395	152.4	67.42	56.4	0.585	23.34	76.10	130.1
F	0.841	66.12	136.9	67.92	50.32	170.3	108.9	1.241	4.092	169.6	76.88	22.08
G	91.60	7.885	69.76	0.822	1.440	0.721	62.39	109.3	1.154	0.214	21.23	129.3
H	1.181	25.57	87.19	0.407	0.537	0.433	19.23	0.785	38.79	27.84	13.67	0.370

NSF. 2:

NSF. 3:

NSF. 4:

plate No. 3.

	1	2	3	4
A	0.565	0.717	0.472	0.172
B	0.316	0.398	21.49	0.436
C	0.351	0.315	0.256	0.398
D	0.210	0.270	0.296	0.242
E	0.701	16.78	0.452	0.167
F	0.733	67.28	0.422	0.157
G	2.103	0.217	0.196	0.165
H	119.8	0.208	0.294	0.164

NSF. 3:

NSF. 4:

Further 5x dilution of a few

	1	2	3	4	5	6	7
A	82.22	51.06					
B	81.99	41.45					
C	71.57	53.15					
D	52.31	57.41					
E	40.75	68.18					
F	58.59	0.150					
G	55.23	0.140					
H	42.75	0.151					

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Proje  
Exp

	1	2	3	4	5	6	7	8	9	10	11	12
A	1a1		1a3		1a5	1a6		1aA	1aB	1aC	1b1	1b2
B			1b5		1b7	1b8		1b10		1b12	1b13	1b14
C	1b15	1b16			1b19	1b20	1b20*	1b23	1b24		2a1	
D	2a3						2a9					
E		2a16		2a18		2b1	2b2	2b3		2b5		2a14
F			2b11	2b12		2b16	2b17				3a2	3a3
G		3a5	3a6	3a7	3a8	3a9					3b5	3b6
H	3b7			3b9	3b10			3b16				4a2

ISF. 3:  
ISF. 4:

plate No. 2

	1	2	3	4	5	6	7	8	9	10	11	12
A	4a3	4a4					4a9		4a11			4aB
B		4aD	4aX	4aY	4aZ							4b8
C		4b10		4b12								
D			5a5			5a8				5a12	5a13	5a14
E	5a15					5b2	5b3				5b8	5b9
F		5b12	5b13	5b14		5b16	5b17			5b22	5b23	
G	5b26		5b29				6a4	6aA				6b2
H			6b5									

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Run Time: 00:29:58

Time: 00:00:00-00:29:58

ISF. 3:  
ISF. 4:

plate No. 3

ISF. 3:  
ISF. 4:

Further rx dilution of a few sample

	1	2	3	4
A				
B				
C				
D				
E				
F		7aA		
G				
H	7a8			

	1	2	3	4	5	6	7	8	9
A	1b1	3b7							
B	1b5	3b11							
C	1b7	5b13							
D	1b20*	5b16							
E	2b5	5b22							
F	2b17								
G	3a3								
H	3b6								

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FI: 0.000 - 1000.000

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Time:

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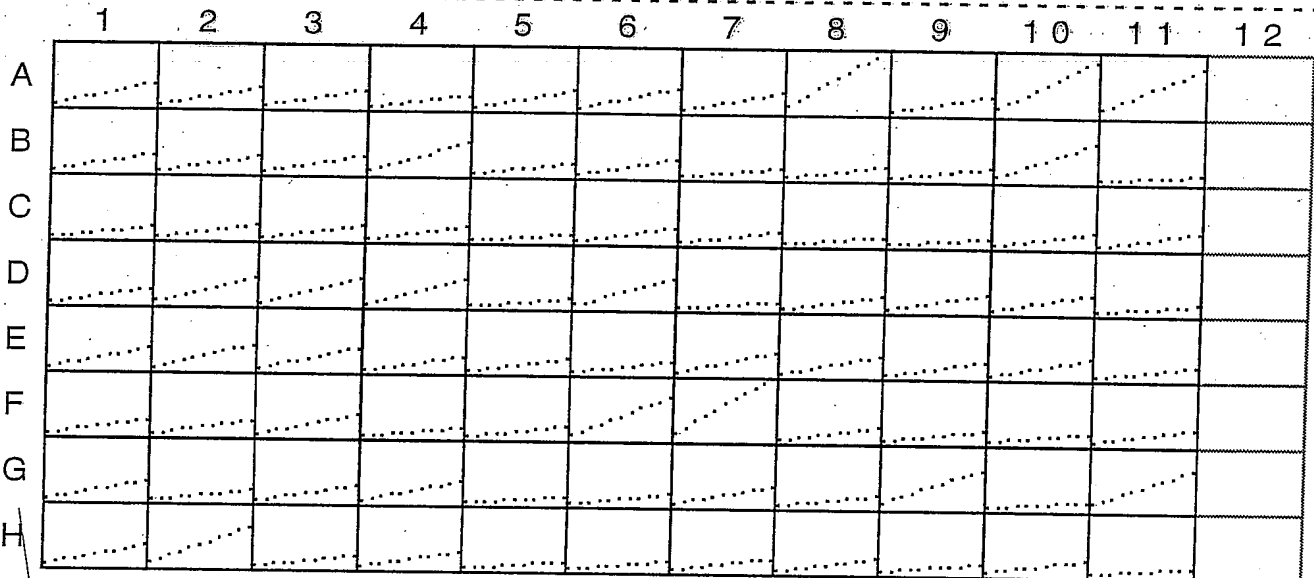
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Experiment Title

Repeat alls Assay for those with too high reading (with red numbering) of yesterday's experiments.

Dilute 5x of the solution used for yesterday's Assay, Use 1.5 ml for each assay.



Read Interval: 00:02:00

FI: 0.000 - 1000.000

Run Time: 00:20:00

Time: 00:00:00-00:20:00

	1	2	3	4	5	6	7	8	9	10	11	12
A	16.89	13.90	13.04	9.804	14.04	16.44	13.71	44.92	13.02	39.14	33.97	
B	13.21	11.84	13.26	24.49	9.566	13.41	7.184	8.893	8.579	27.22	4.855	
C	7.982	10.47	9.789	10.41	5.600	11.05	8.823	5.965	5.442	9.739	20.62	
D	11.24	19.73	20.59	21.02	6.334	22.91	5.853	9.915	12.79	13.22	6.288	
E	15.40	18.74	16.94	11.34	9.949	9.317	16.84	14.69	10.74	13.22	8.365	
F	11.35	11.34	16.02	7.297	9.181	31.19	47.35	9.792	8.264	7.410	10.49	
G	14.42	8.307	12.05	16.12	5.499	7.863	14.70	8.251	27.26	5.649	28.11	
H	15.65	28.87	8.750	12.60	5.594	7.089	9.247	9.937	6.743	10.10	5.212	

These figures are generally lower than expected (or yesterday's reads)

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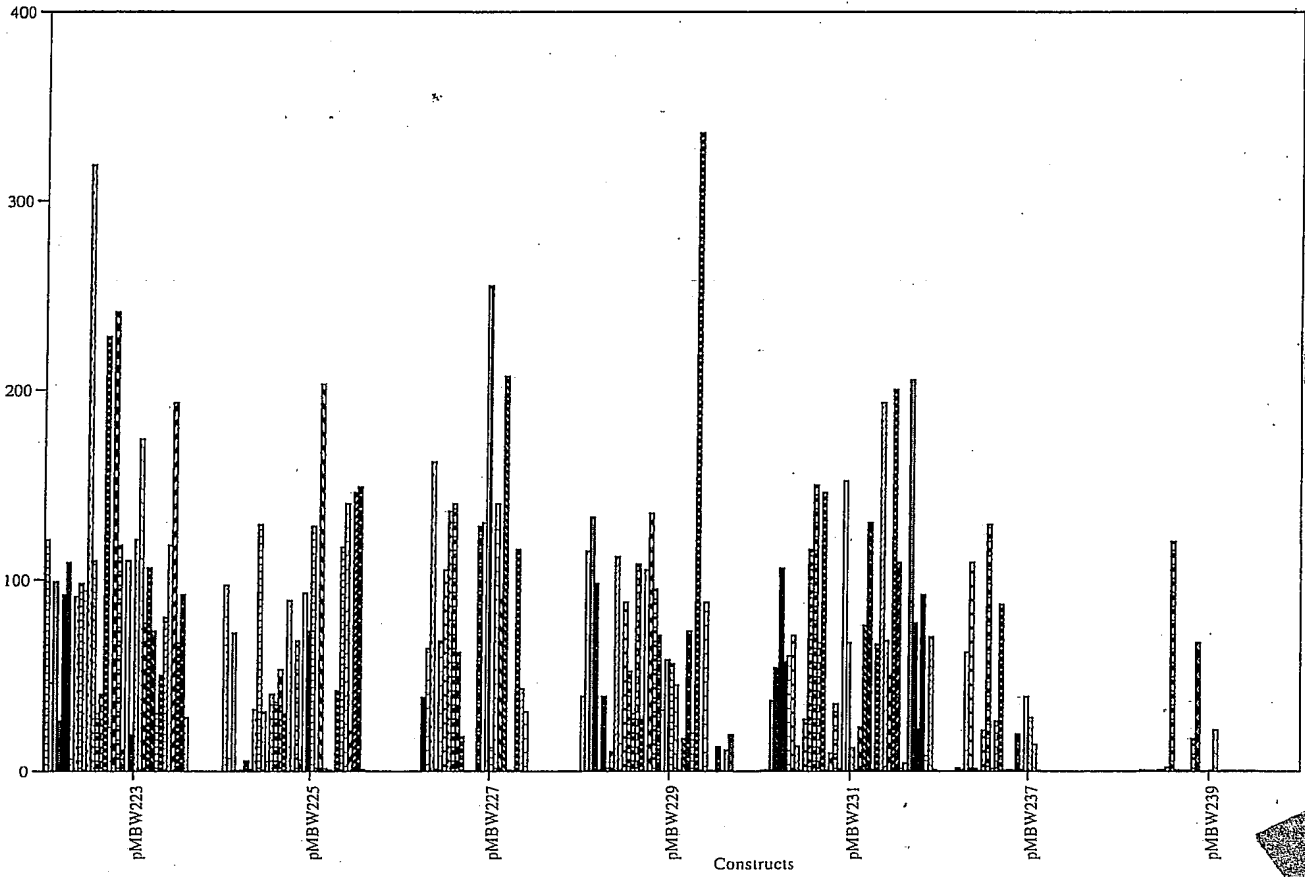
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